Dicumarol Therapy and Platelet Turnover

By E. A. MURPHY, M.D., AND J. F. MUSTARD, M.D., PH.D.

The fate of the blood platelets is not as yet completely known. Since it has been established, however, that they are used in the formation of intravascular white thrombi, factors that decrease the tendency of platelets to adhere and clump might be expected to produce longer survival. Dicumarol (Dicoumarol) in sufficient doses in man and in animals delays the onset of platelet clumping as measured by in vitro tests. It is important to determine whether these in vitro changes reflect phenomena in vivo, and this is conveniently tested by estimating platelet turnover by in vivo isotope-tagging techniques.

Platelet survival was accordingly studied in two groups of atherosclerotic male subjects, one of which was untreated, while the other received dicumarol in amounts sufficient to delay platelet clumping and diminish platelet adhesiveness.

Methods

Sixty male subjects between the ages of 34 and 75 years were studied: 29 were given dicumarol or related drugs, 31 were used as controls. The two groups were comparable in age and clinical manifestations, although not allocated in a strictly random fashion. Thus there may be some bias, but this is believed to be unimportant. All of them had sustained coronary, cerebral, or peripheral vascular complications, the most recent incident being not less than three months previously. All the treated subjects had been receiving dicumarol for at least three weeks before the platelet survival was studied.

Coagulation Tests

Whole-blood clotting time, prothrombin time, plasma thromboplastin time (activity of dilute plasma in place of serum in the thromboplastin generation test), platelet count, platelet adhesive index, and platelet clumping time were determined by methods previously described.

Platelet Survival

This was estimated by the technique of Leeksma and Cohen. The platelets were tagged with diisopropyl fluorophosphate, which contains phosphorus as P32 (DFP32). It forms a linkage, reported to be irreversible, with all esterases. Some modifications of their method were introduced.

The DFP32 (specific activity 150 to 250 μc./mg. DFP*) was dissolved in propylene glycol. Each subject was given a dose of from 70 to 100 μc. intramuscularly. Blood samples were collected from the fasting patients in the morning on the day after injection and daily thereafter (with the exception of Sundays) for 9 to 11 days. Forty-five ml. of blood was added to 5 ml. of a disodium ethylenediaminetetraacetate (EDTA) solution (2.6m. EDTA, 0.33 Gm. sodium chloride, 100 ml. distilled water) in a silicone-coated centrifuge tube at 4°C. The blood sample was centrifuged at 1000 r.p.m. (RCF 225) in a M.S.E. major refrigerated centrifuge at 4°C. for 10 minutes. The upper two-thirds of the supernatant plasma was then transferred to a silicone-coated glass centrifuge tube. The blood remaining in the original tube was resuspended by shaking and again centrifuged at 1000 r.p.m. for 10 minutes. The supernatant plasma from this preparation was added to that from the first. The pooled platelet-rich plasma was then centrifuged at 2600 r.p.m. (RCF 1540) at 4°C. for 14 minutes. The supernatant was discarded and the platelet button gently transferred to a silicone-coated glass centrifuge tube. The blood remaining in the original tube was resuspended by shaking and again centrifuged at 1000 r.p.m. for 10 minutes. The supernatant plasma from this preparation was added to that from the first. The pooled platelet-rich plasma was then centrifuged at 2600 r.p.m. (RCF 1540) at 4°C. for 14 minutes. The supernatant was discarded and the platelet button gently transferred to a silicone-coated glass centrifuge tube and suspended in 20 ml. of a saline-EDTA solution for washing (one part of the above EDTA solution, nine parts 0.15 per cent saline). Frequently, a small red-cell deposit was found at the bottom of the platelet button. When this occurred only the upper part of the platelet button, which was grossly free of red blood cells, was transferred to the wash solution. The platelet suspension was centrifuged at 2600 r.p.m. at 4°C. for 14 minutes and the platelet suspension centrifuged at 1000 r.p.m. at 4°C. for 14 minutes and the platelet suspension centrifuged at 1000 r.p.m. at 4°C. for 14 minutes and the platelet button was gently suspended in 1.0 to 2.0 ml. of 0.15 per cent saline. A platelet count was done on this suspension. If the number of red cells

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was greater than 1 per 5,000 platelets, the platelet suspension was diluted to 20 ml. with saline and the platelet suspension centrifuged at 1000 r.p.m. for 10 minutes at 4°C. The supernatant was then transferred to a centrifuge tube and centrifuged at 2600 r.p.m. for 14 minutes. The supernatant was discarded and the platelet button suspended in 1 to 2 ml. of saline and the platelet count repeated. The platelet count and fluid volume of each final platelet suspension were determined, and this suspension then transferred to a stainless steel planchette and the material dried in an oven at 90°C.

The time for 400 counts was determined using a well counter. The radioactivity was corrected for the background radiation level and physical decay, and expressed as counts per minute per 10^9 platelets. The method of calculating platelet survival follows.

Calculation of Platelet Survival

The mathematical pattern of the disappearance of platelets from the circulation may conceivably be determined by factors of internal economy of the platelet or the demands of external economy, or some combination of the two (see Discussion). We regard this matter as open and have it at present under investigation. Leekso and Cohen^4 have found a linear fall-off with DFP^32, which would suggest that survival is normally distributed about a mean, with a small coefficient of variation. In our subjects, the fall-off seems to conform more closely to an exponential decay, and our results have been computed accordingly by fitting a regression line to the logarithms of the activities by the method of least squares. It seems probable that any error arising from assuming the wrong mathematical pattern will be introduced alike to both groups, which may then be compared without much danger of bias. Examination of the data has in fact shown an excellent correlation between the two estimates of mean survival made by using the two mathematical patterns.

From simple mathematical considerations, the mean survival of the cells is given by the time interval required for the circulating count to fall by the fraction \(\left(1 - \frac{1}{e}\right)\), that is, by 63 per cent, and this in days divided into the mean platelet count per cubic millimeter is the platelet turnover in platelets per cubic millimeter per day, assuming that the platelet count is in a steady state. Since many biological quantities are measured in half-lives, it should be added that the mean survival can be calculated by dividing the half-life by \(\log_e 2\) which is 0.69315.

Statistical Considerations

Mention of the distribution of some of the variates has been made in an earlier paper.\(^3\) Several more variates are involved in this study, and we may summarize our findings as follows: (a) Prothrombin time, platelet count, platelet adhesive index, and platelet survival are normally distributed. (b) Whole-blood clotting time and platelet clumping time are log normally distributed. (c) Plasma thromboplastin time is harmonic normally distributed.

Values are calculated with the appropriate transformations; means given in table 1 have been translated back into the original scale of measurements and therefore represent arithmetic, geometric, and harmonic means respectively.

Results

Coagulation Tests

The mean values for the whole-blood clotting time, prothrombin time, plasma thromboplastin time, and platelet clumping time were significantly longer in the treated than in the untreated group (table 1). The mean platelet adhesive index was significantly lower in the treated group. None of these differences was affected by age correction.

Platelet Turnover

The mean platelet turnover in the subjects receiving dicumarol therapy was significantly less than that in the subjects not receiving dicumarol therapy (\(P < 0.001\)), table 2.\(^*\) The significance of the differences was unaffected when family history and age were taken into consideration. There was no significant difference between the mean platelet counts in the two groups.

Relationship Between Mean Platelet Survival and Coagulation Tests

These relationships represented as correlation coefficients are given in table 3. The results for the treated and untreated cases are similar; the pooled value given in table 3. The results for the treated and untreated cases are similar; the pooled value given in table 3. The results for the treated and untreated cases are similar; the pooled value given in table 3. The results for the treated and untreated cases are similar; the pooled value given in table 3.

*Treating platelet destruction as random necessarily leads to a shorter estimate of the mean survival than that obtained on the same set of results by treating it as "linear." Computation of our results on the "linear" model gives a mean value of 10.04 ± 0.27 days for the untreated and 11.70 ± 0.28 days for the treated patients, a highly significant difference statistically (\(P < 0.001\)). The values obtained by both these mathematical models are of the same magnitude as those obtained by various other investigators using the corresponding methods.

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Coagulation Tests

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean whole-blood clotting time (min.)</th>
<th>Mean prothrombin time (sec.)</th>
<th>Mean plasma thromboplastin time (sec.)</th>
<th>Mean platelet clumping time (sec.)</th>
<th>Mean platelet adhesive index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>12.6</td>
<td>14.1</td>
<td>11.6</td>
<td>308</td>
<td>1.25</td>
</tr>
<tr>
<td>Treated</td>
<td>18.9</td>
<td>31.0</td>
<td>20.2</td>
<td>452</td>
<td>1.01</td>
</tr>
</tbody>
</table>

Significance of differences between means

\[ P <<0.001*** \]

The significance of the differences is unaffected by age correction.

Table 2

Platelet Turnover

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of subjects</th>
<th>Mean age (years)</th>
<th>Mean platelet count no./cu. mm.</th>
<th>Platelet half-life (days)</th>
<th>Platelet turnover no./cu. mm. of blood/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>31</td>
<td>55.3</td>
<td>222,000</td>
<td>2.86</td>
<td>58,200</td>
</tr>
<tr>
<td>Treated</td>
<td>29</td>
<td>49.9</td>
<td>201,000</td>
<td>3.84</td>
<td>38,500</td>
</tr>
</tbody>
</table>

Significance of differences between age-corrected means

\[ t \equiv \sqrt{F} \]

\[ P <0.1 \]  \[ <0.001*** \]  \[ <0.001*** \]

Discussion

These results show that the platelets of atherosclerotic patients receiving dicumarol therapy have a longer survival than those of comparable untreated atherosclerotic subjects. Since the mean platelet counts were similar, this indicates that subjects receiving dicumarol are producing and using fewer platelets than the untreated group. This may prove important in the study of factors that regulate platelet production. As to the fate of the circulating platelet, we do not know whether this is determined by the external or the internal economy of the platelet or by a combination of the two. If it is regulated by the internal economy, then we have to suppose that dicumarol prolongs the spontaneous survival of the platelet. It seems much more plausible to suppose that the platelet survives longer because of the lessened demands of the external economy. It is known that the converse is true: acceleration of clotting by the parenteral administration of serum, thrombin, or tissue thromboplastin produces a precipitous fall in the circulating platelet count. Furthermore, the positive relationship between the extent of the anticoagulation and the prolongation of platelet half-life can be more easily reconciled with the view that it is the external economy of the platelet that is being changed by dicumarol. The alternative argument, that the changes in coagulation are secondary to changes in the internal economy of the platelet, does not appear reasonable in the light of evidence so far available. It must be pointed out, however, that the results of the present study obtained in atherosclerotic
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Table 3
Correlations Between Platelet Half-Life and Other Variables

<table>
<thead>
<tr>
<th>Group</th>
<th>Reciprocal of plasma thromboplastin time (factor IX)</th>
<th>Prothrombin time</th>
<th>Platelet adhesive index</th>
<th>Platelet count</th>
<th>Log platelet clumping time</th>
<th>Log whole-blood clotting time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (31)</td>
<td>−0.38†</td>
<td>+0.26</td>
<td>−0.32</td>
<td>−0.05</td>
<td>+0.16</td>
<td>+0.04</td>
</tr>
<tr>
<td>Treated (29)</td>
<td>−0.21</td>
<td>+0.45†</td>
<td>−0.42†</td>
<td>+0.25</td>
<td>+0.34</td>
<td>−0.01</td>
</tr>
<tr>
<td>Pooled (within)</td>
<td>−0.30†</td>
<td>+0.35‡</td>
<td>−0.36‡</td>
<td>+0.10</td>
<td>+0.25</td>
<td>+0.02</td>
</tr>
</tbody>
</table>

†P < 0.05
‡P < 0.01

Male subjects may not be universally applicable. Such subjects commonly have altered endothelial surfaces, which are known to promote platelet deposition. In subjects with a more normal arterial tree the main factor governing the survival of platelets may prove to be the internal economy.

Nevertheless, the results show that, when therapy has been directed at decreasing platelet adhesiveness and prolonging platelet clumping in vitro, platelet use is correspondingly decreased in vivo. In view of the basic role of the platelet in thrombosis and hemorrhage, these in vitro and in vivo changes in platelet function are probably of importance in elucidating the criteria for the effective control of dicumarol therapy.

The relationship between platelet survival and the various clotting tests produced some interesting and unexpected results. It might have been predicted that platelet survival would have been more closely related to the platelet clumping time than to the prothrombin time. It is possible, however, that the converse found here is a statistical artifact. The calculation of a correlation coefficient presupposes in theory that the two variates are being measured without experimental error: any experimental error gives an underestimate of closeness of the true relationship, and the larger the error relative to the scatter of the variates about their means, the greater this underestimate will be. It has been pointed out above, however, that, in this study, treatment has been directed toward maintaining the platelet clumping time within a narrowly defined therapeutic range, whereas the ranges of the other clotting tests have not been primarily restricted. Thus, there is a greater tendency for the correlation of platelet survival with the platelet clumping time to be underestimated.

The best correlation is with the adhesive index despite the fact that this test has previously been shown to have a relatively higher experimental error than the platelet clumping test. It may be that the adhesiveness of platelets is more intimately concerned with their destruction than is their cohesiveness. It seems probable that platelet clumping is not a normal phenomenon in circulating blood, but is primarily concerned with the plugging of damaged vessels. If physiological platelet destruction involves their adhesion to surfaces, then the more glutinous the platelet the more readily it will cease to circulate.

There is evidence that small doses of dicumarol make platelets more adhesive, but do not shorten the prothrombin time (personal communication from L. Horlick). It thus appears that the dosage-adhesive-index relationship may be biphasic, while the dosage-prothrombin time is not. Thus, a diminished adhesive index must necessarily be associated with a prolonged prothrombin time, whereas a prolonged prothrombin time need not be associated with a decreased platelet adhesive index. We must then expect that when dicumarol dosage is directed toward decreasing the adhesive index, thus ensuring that all readings are made in one phase, rather better correlation will be obtained between the prothrombin time and tests of platelet survival than if treatment is directed toward prolong-
ing the prothrombin time. In this study, treatment has been directed toward the former, whereas in our earlier study, it was directed toward the latter object.3

These factors may in part explain why the prothrombin time shows a better correlation with estimates of platelet survival than previous studies might have led us to believe.

Summary

Platelet survival and turnover were studied by the use of diisopropyl fluorophosphate, which contains phosphorus as P32 (DFP32) in atherosclerotic male subjects. Twenty-nine were given dicumarol in doses sufficient to diminish platelet function in vitro and 31 were used as controls. Platelet survival was prolonged and platelet turnover was decreased by dicumarol therapy. Of the coagulation tests, the platelet adhesive index showed the best correlation with platelet turnover. These findings indicate that platelet function in vitro and in vivo is diminished by adequate dicumarol therapy. Evidence is presented that the survival of the platelet, at least in the atherosclerotic subject, is largely determined by factors external to the platelet, among which the activity of the coagulation mechanism is important.

Acknowledgment

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References

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