Clearing Factor Inhibitor in Human Atherosclerosis


With the technical assistance of J. A. Peters, D.M.T.

A reduction in heparin-activated clearing factor has been demonstrated in individuals with atherosclerosis or conditions related to it. The recent isolation from normal plasma of an inhibitor to clearing factor suggested that the reduction described might be due to an increased level of inhibitor. This possibility was therefore investigated by determining the inhibitor effect of the plasma of atherosclerotic, diabetic, and nephrotic individuals on the in vitro clearing activity of post-heparin plasma.

The role of heparin in the pathogenesis of human atherosclerosis still requires considerable clarification. The fact that heparin is capable of altering the blood lipid picture,1-4 and that atherosclerosis is associated with increased blood lipid levels,5-7 naturally directed investigation to heparin deficiency as a contributing factor. It has been reported that the blood heparinoids are reduced in atherosclerotic subjects8,9 and also that heparin administered therapeutically exerts a favorable influence on persons with coronary atheroma.10,11 Heparin, however, does not act directly on blood lipids, its action being brought about by the elaboration of clearing factor,12 which is capable of clearing fat emulsions both in vivo and in vitro. It has been shown that the elaboration of clearing factor is reduced in individuals with atherosclerosis,13,14 small doses of intravenous heparin producing less clearing following a fat meal in this group. The evidence that clearing factor is present in normal plasma15,16 and can be extracted from normal tissues,17 increases in importance any possible disturbance of the clearing reaction manifested by the atherosclerotic individual. Disturbance of the physiology of fat transport resulting from a reduced clearing rate may therefore be a factor in the elevated lipid levels of the atherosclerotic individual. Further, in experimental nephrosis and diabetes,18,19 there is evidence of a reduced clearing factor response to heparin and both these conditions are well known precursors of atherosclerosis.

Recently it was found20,21 that normal plasma contained a substance, probably a glycoprotein, capable of inhibiting clearing factor. The decreased clearing following small doses of heparin in older persons and atherosclerotic subjects in particular,13,14 could well be related to a higher level of inhibitor in these groups. The possibility of an increased level of clearing factor inhibitor in individuals with atherosclerosis and related conditions was therefore considered worth investigating. Indeed recently Klein and Lever22 reported that idiopathic hyperlipemia was associated with increased clearing factor inhibitor activity and Angervall and Hood20,23 made some inconclusive observations on its presence in atherosclerotic subjects.

In this paper the clearing factor inhibitor activity of the plasma of atherosclerotic, diabetic, and nephrotic subjects is determined and compared with that of younger student controls and with controls of equivalent age.

Methods

The plasma of 4 groups of individuals were investigated for their clearing factor inhibitor activity. The control group consisted of 16 normal medical students varying in age from 19 to 27 years (mean = 22.0). The second group was an older control group consisting of 14 subjects varying in age from 35 to 72 years (mean = 48.5). They were mostly convalescent hospital patients with no evidence of atherosclerosis or related conditions. The third group consisted of 17 individuals of equivalent age, but with definite evidence of coronary atherosclerosis. They were either patients with a history of myocardial infarction, at least 3 months before, or of severe angina pectoris. Their age varied from 40 to 72 years (mean
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Table 1.—In vitro, Clearing Rate (k) of Post-Heparin Plasma When Mixed with Untreated Plasma (Student, Age Control, Atherosclerotic, Diabetic, and Nephrotic)

<table>
<thead>
<tr>
<th>Date</th>
<th>Student</th>
<th>Age control</th>
<th>Atherosclerotic</th>
<th>Diabetic</th>
<th>Nephrotic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age</td>
<td>k</td>
<td>Age</td>
<td>k</td>
<td>Age</td>
</tr>
<tr>
<td>19/8</td>
<td>26</td>
<td>.123</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>.122</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>10/9</td>
<td>19</td>
<td>.143</td>
<td>40</td>
<td>.090</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>.153</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16/9 (1)</td>
<td>24</td>
<td>.168</td>
<td>49</td>
<td>.138</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>.122</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16/9 (2)</td>
<td>23</td>
<td>.171</td>
<td>39</td>
<td>.098</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>72</td>
<td>.105</td>
<td>—</td>
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<tr>
<td>23/9 (1)</td>
<td>22</td>
<td>.168</td>
<td>65</td>
<td>.160</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>—</td>
<td>—</td>
<td>72</td>
</tr>
<tr>
<td>23/9 (2)</td>
<td>21</td>
<td>.165</td>
<td>40</td>
<td>.132</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50</td>
<td>.147</td>
<td>—</td>
</tr>
<tr>
<td>24/9</td>
<td>20</td>
<td>.158</td>
<td>37</td>
<td>.140</td>
<td>53</td>
</tr>
<tr>
<td>30/9 (1)</td>
<td>21</td>
<td>.114</td>
<td>70</td>
<td>.130</td>
<td>48</td>
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<tr>
<td></td>
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<td></td>
<td>—</td>
<td>—</td>
<td>58</td>
</tr>
<tr>
<td>30/9 (2)</td>
<td>22</td>
<td>.100</td>
<td>—</td>
<td>—</td>
<td>55</td>
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<td></td>
<td></td>
<td></td>
<td>—</td>
<td>—</td>
<td>58</td>
</tr>
<tr>
<td>8/10 (1)</td>
<td>20</td>
<td>.180</td>
<td>41</td>
<td>.220</td>
<td>53</td>
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<td></td>
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<td>—</td>
<td>—</td>
<td>26</td>
</tr>
<tr>
<td>8/10 (2)</td>
<td>20</td>
<td>.270</td>
<td>56</td>
<td>.130</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>—</td>
<td>—</td>
<td>46</td>
</tr>
<tr>
<td>8/10 (3)</td>
<td>20</td>
<td>.145</td>
<td>49</td>
<td>.154</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>—</td>
<td>—</td>
<td>30</td>
</tr>
<tr>
<td>21/10 (1)</td>
<td>20</td>
<td>.310</td>
<td>37</td>
<td>.255</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>—</td>
<td>—</td>
<td>50</td>
</tr>
<tr>
<td>21/10 (2)</td>
<td>21</td>
<td>.330</td>
<td>35</td>
<td>.325</td>
<td>40</td>
</tr>
</tbody>
</table>

= 54.3). The fourth group consisted of diabetic subjects covering a fairly large age range (17 to 80 years). In addition 2 patients with nephrotic syndrome were investigated. Blood was collected from all these individuals into chilled oxalated tubes, and the plasma was separated and kept at 4 C. until clearing factor inhibitor activity was tested. This was done within 4 hours of collection.

The source of lipemia-clearing factor used for the inhibitor studies was plasma taken from normal medical students after they were given heparin. On each day 1 or more students was given intravenously 1,000 units of heparin and 15 minutes later 10 ml. of blood were removed. It was placed in chilled oxalated tubes and the plasma was separated and kept at 4 C. until used later the same day. Since this procedure was repeated on each day, a different source of clearing factor was used on different days. In order to determine inhibitor activity 0.25 ml. of the active post-heparin plasma was mixed with 0.25 ml. of the plasma to be tested and the resulting clearing activity was determined. The student controls were tested at the same time and under the same conditions as the other 3 groups, as far as possible a representative of all groups being incorporated.
TABLE 2.—Reduction in "in vitro" Clearing Rate Caused by Age Control, Atherosclerotic and Diabetic Plasma Relative to the Student Control in Each Series. Relative Inhibitor Activity Expressed as

<table>
<thead>
<tr>
<th>Age control</th>
<th>Atherosclerotic</th>
<th>Diabetic†</th>
</tr>
</thead>
<tbody>
<tr>
<td>39.2</td>
<td>−39.8*</td>
<td>36.6</td>
</tr>
<tr>
<td>17.9</td>
<td>58.0</td>
<td>22.8</td>
</tr>
<tr>
<td>42.7</td>
<td>17.9</td>
<td>−29.7*</td>
</tr>
<tr>
<td>38.6</td>
<td>40.3</td>
<td>3.4</td>
</tr>
<tr>
<td>4.8</td>
<td>9.5</td>
<td>−8.3*</td>
</tr>
<tr>
<td>20.0</td>
<td>29.8</td>
<td>25.0</td>
</tr>
<tr>
<td>10.9</td>
<td>27.2</td>
<td>65.6</td>
</tr>
<tr>
<td>11.4</td>
<td>19.3</td>
<td>11.1</td>
</tr>
<tr>
<td>−14.0*</td>
<td>−38.0*</td>
<td>−10.3*</td>
</tr>
<tr>
<td>−22.2*</td>
<td>18.0</td>
<td>−</td>
</tr>
<tr>
<td>51.8</td>
<td>−20.0*</td>
<td>−</td>
</tr>
<tr>
<td>−6.2*</td>
<td>10.0</td>
<td>−</td>
</tr>
<tr>
<td>17.7</td>
<td>11.1</td>
<td>−</td>
</tr>
<tr>
<td>1.5</td>
<td>3.4</td>
<td>−</td>
</tr>
<tr>
<td>−</td>
<td>31.2</td>
<td>−</td>
</tr>
<tr>
<td>−</td>
<td>34.2</td>
<td>−</td>
</tr>
<tr>
<td>−</td>
<td>26.0</td>
<td>−</td>
</tr>
<tr>
<td>15.3 ±5.7</td>
<td>14.0 ±6.3</td>
<td>12.9 ±8.9</td>
</tr>
</tbody>
</table>

* Greater clearing rate than respective student controls expressed as negative inhibition.
† Only diabetic subjects over 35 years included.

in the series for each day. It was thus possible on each day or within each series (where 2 sources of clearing factor were used) to compare the amount of inhibition produced by the student control with that produced by the other patients. Thus the clearing factor inhibitor activity of the control, atherosclerotic, and diabetic groups could be compared with the student control.

Lipemia-clearing factor inhibitor activity. The clearing factor activity of the mixture of active post-heparin plasma with the plasma to be investigated was determined by the method of Grossman* as modified by Day and Peters.* In vitro reduction in the turbidity of the artificial emulsion, when mixed with the plasma mixture, was read immediately after mixing and at 5, 10, 20, 30, 45, and 60 minutes thereafter.

The graph of log, D (optical density) against time was approximately linear in all cases, indicating that the rate of clearing at any instant was proportional to the optical density at that instant, or in other words that the optical density decreased with time according to the negative exponential decay law. Mathematically, this may be expressed as follows:

\[ D_t = D_0 e^{-kt} \]

\[ \frac{dD_t}{dt} = -k \cdot D_t \cdot e^{-kt}; \]

\[ \frac{1}{D_t} \frac{dD_t}{dt} = d \cdot \log, D_t, = -k, \]

where \( D_t \) is the optical density at time \( t \) (\( D_0 \) at \( t = 0 \)). The constant \( k \) is the proportional clearing rate, and is the appropriate index of clearing factor activity.

The \( k \)-values were determined in each case by plotting \( \log, D_t \) against \( t \), fitting a straight line to the plot, and measuring the slope of the line. For comparison of the \( k \)-value for a test individual (age control, atherosclerotic etc.) with that for a student, the following index was considered to be more appropriate than the simple difference of \( k \) values, namely:

\[ I = \frac{k(\text{student}) - k(\text{test group})}{k(\text{student})} \times 100\% \]

since it was found that the difference \( k_s - k_t \) depended to some extent on the clearing factor activity of the serum used, as indicated by the \( k \)-value. This index was taken as a measure of clearing factor inhibitor activity in the test individual (relative to control).

RESULTS

Table 1 gives the in vitro clearing rate \( (k) \) of post-heparin plasma when mixed with untreated plasma from students, age control, atherosclerotic, diabetic, and nephrotic subjects. On each day, (or in each series if more than one series was performed on the day) the same source of clearing factor was used and the student inhibitor activity was determined at the same time and under the same conditions as the other patients studied on that day. It was possible therefore to consider the student control as having zero inhibitor activity and to relate the inhibitor activity of the test subjects to their respective student control on this basis. In table 2 this has been done and the relative inhibitor activities \( (I) \) of the age control, atherosclerotic, and diabetic subjects have been calculated and compared.

It is apparent from tables 1 and 2 that there is greater clearing factor inhibitor activity in all 3 test groups than in the student group. In the age control series, 11 of the 14 subjects have more inhibitor activity than their respective student controls. In the remaining 3 the "negative inhibition" is simply an expression of greater inhibitor activity in the student than in that age control. The mean for \( I \), with its estimated standard error, is 15.3 ± 5.7, an increased
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inhibitor activity with age that is statistically significant \( p < 0.02 \).

In the atherosclerotic group 14 of the 17 patients demonstrated more inhibitor activity than the respective student controls. The mean value for \( I \) for this group was 14.0 \( \pm \) 6.3, this difference again being statistically significant \( p < 0.05 \). When the atherosclerotic group was compared with the age control group, i.e., with normal individuals of similar age, no significant difference in inhibitor activity was detected. It would seem that the difference in inhibitor activity between the student and atherosclerotic groups may be explicable entirely on an age basis and not related to atherosclerosis as such.

In the diabetic group, when the younger diabetics (<35 years) are excluded, the mean inhibitor activity \( I \) is 12.9 \( \pm \) 8.9. Because of the large variance this value is not significantly different from the student group, but it is of the same order as the other 2 "older groups." No significant difference in inhibitor activity inhibitor exists between the diabetic and the age control or atherosclerotic individuals. Thus no specific increase in inhibitor activity can be attributed to the diabetic state. It is worth noting that in the 3 younger diabetics (26, 17, and 30 years) the inhibitor activity is of the same order as the student controls. Thus when the age factor is eliminated, the increased inhibitor activity in this group is also eliminated.

Definite conclusions cannot be drawn about the 2 nephrotic subjects, but it is interesting that there is inhibitor activity (compared with the student control) in both. However this inhibition is similar in magnitude to that of the age controls investigated on the same day and can therefore be accounted for purely on an age basis.

**Discussion**

It has been found that the plasma of older individuals inhibits lipemia-clearing factor to a greater degree than younger controls. This elevated inhibitor activity with aging is in accord with the recent findings of Nikkilä and Niemi,\(^26\) who have shown heparin to produce less clearing factor in older subjects than in younger controls. The presence of more inhibitor activity as shown here would reduce the post-heparin clearing activity and thus account for this apparently reduced response. In a previous communication\(^4\) we have recorded the fact that protamine elevates the serum total fatty acids in normal students, but has no effect in older age groups. This was interpreted to indicate a deficiency in circulating heparin produced with aging. Hollett and Meng\(^27\) have shown that protamine is capable of inhibiting clearing factor and that its action on lipids may be related partly to this inhibition. The changing response with age of fatty acids to protamine could therefore also be explained in terms of an increased clearing factor inhibitor activity. With the small amount of normal clearing factor already inhibited in the older age groups, the inhibiting effect of protamine would not be able to manifest itself and minimal changes would result. The reduced fat tolerance with increasing age\(^28, 29\) can also be related to the increased inhibitor level. Clearing factor, physiologically concerned with lipid removal following a fat meal, is prevented from adequately doing so by the increased inhibitor activity and the fat level is able to rise to a higher level in the older age groups. The findings of Block, Barker, and Mann\(^13\) that a reduced sensitivity to heparin exists in atherosclerotic individuals can also be interpreted in terms of the findings of this present paper. In actual fact these workers used a very much younger control group and the reduced clearing by heparin, attributed by them to atherosclerosis, was possibly no more than an age difference. Such an age difference probably relates to an increased inhibitor level and not to a reduced sensitivity to heparin as was supposed. The difference due to atherosclerosis, although supported by Oliver and Boyd,\(^14\) was not confirmed by others.\(^30, 31\)

Whether there is a difference in clearing factor inhibitor activity specific to individuals with atherosclerosis is doubtful. Certainly no evidence to that effect was obtained here. It is relevant to raise the difficulty in selection of nonatheromatous controls, the problem of satisfactorily excluding individu-
als with occult atheroma being widely appreciated. Nevertheless Angervall and Hood,20, 21 while obtaining some evidence for increased inhibitor activity with atheroma, were unable to confirm this and they concluded that no real difference exists. The possibility remains however, that one influence of aging on the atherosclerotic process is the demonstrated increase in clearing factor inhibitor with its consequent effect on blood lipid level.

Regarding the inhibitor activity of the diabetic and nephrotic groups, little further need be said. No difference specific to the respective conditions was noted, the only change being one associated with the age of the individual and similar in magnitude to the age control group.

**Summary**

The inhibitor effect of the plasma of atherosclerotic, diabetic, and nephrotic individuals on in vitro clearing activity of post-heparin plasma was determined and compared with that of student controls and older controls. A significant increase in inhibitor activity was shown to exist between the older control group and the student group. A similar increase in inhibitor activity was demonstrated when the atherosclerotic group was compared with the student group, but no difference existed when compared with the control group of similar age. An increase in inhibitor activity was found in both the diabetic and nephrotic group, but again no difference when the age effect was eliminated. It was considered that an increased level of clearing factor inhibitor results from aging, but that no increased activity occurred with atherosclerosis, diabetes, or nephrosis, other than that accountable for by age.

**Acknowledgment**

We are indebted to Dr. H. R. Gilmore of the Department of Medicine for the selection of much of the clinical material and for the consequent blood sampling. To Mr. A. E. Bowey of the Department of Pharmacy for preparing the emulsion substrate used for clearing factor determinations, to Miss M. Redway for technical assistance, and to the many students and patients who cooperated in this investigation we would also like to record our thanks.

**Summario in interlingua**

Esseva determinate le effecto inhibitori que plasma ab individuos atherosclerotic, diabetic, e nephrotic exerce in vitro super le activitate clarificatori in plasma post-heparinic. Le resultatos esseva comparate con le corrispondente effecto exercite per plasma ab individuos de controlo (1) de etate universitari e (2) de etate plus avantiate. Esseva constatate un augmento significative in le activitate inhibitori in le caso del gruppo de controlo a etate avantiate in comparation con le gruppo de studentes universitari. Un simile augmento del activitate inhibitori esseva demonstrate in le caso del gruppo atherosclerotic in comparation con le gruppo de studentes, sed nulle differentia existeva inter le gruppo atherosclerotic e un gruppo de controlo di simile etates. Esseva constatate un augmento del activitate inhibitori in le gruppo diabetic e etiam in le gruppo nephrotic, sed etiam in iste caso le differentia dispereva si tosto che le effecto del etate esseeva eliminate. Es formulate le opinion que un augmento del activitate del factor clarificatori resulta ab le avantiamiento del etate sed que nulle tal augmento occurre in casos de atherosclerosis, de diabete, o de nephrosis, excepte le augmento que es explicable per le etate del subjectos.

**References**


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