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### An Acute Effect of Cigarette Smoking on Platelet Function: A Possible Link Between **Smoking and Arterial Thrombosis** PETER H. LEVINE

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# An Acute Effect of Cigarette Smoking on Platelet Function

## A Possible Link Between Smoking and Arterial Thrombosis

By Peter H. Levine, M.D.

#### SUMMARY

In a controlled, double blind study, the smoking of a single cigarette has been shown to increase the platelet's response to a standard aggregating stimulus. This phenomenon appears to be specifically related to the inhaling of tobacco smoke; it does not follow the smoking of lettuce leaf filled cigarettes. The platelet effect seems independent of the rise in plasma free fatty acids which follows cigarette smoking. Smoking-induced potentiation of platelet aggregation may help to explain the increased incidence of arterial thrombi and/or atheromatous plaques in cigarette smokers.

#### **Additional Indexing Words:**

Free fatty acids Platelet aggregation

A RTERIAL THROMBOSIS occurs with increased frequency in cigarette smokers.<sup>1</sup> Myocardial infarction,<sup>2</sup> occlusive peripheral arterial disease,<sup>3</sup> and late occlusion of aortofemoral bypass grafts<sup>4</sup> are examples of this phenomenon. Because the blood platelet appears to play the central role in the initiation of arterial thrombi,<sup>5</sup> definition of the relationship between smoking and platelet function seems important.

The lifespan of platelets has been found to be reduced during periods of heavy smoking.<sup>6</sup> Platelet adhesiveness has been measured in cigarette smokers,<sup>7</sup> and conflicting results have emerged. In two independent studies,<sup>8, 9</sup> platelet aggregation was chronically increased in heavy smokers when compared with a population of non-smokers. Acute effects of smoking on the electrophoretic mobility of platelets was suggested in one of these studies,<sup>9</sup> but no acute effect on platelet aggregation could be shown in either study. That any of the above changes in platelet function are a result of some

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constituent of tobacco smoke remains unproven. A possible specific effect of smoking on platelet aggregation is the subject of this paper.

The measurement of platelet aggregation in vivo suffers from lack of reproducibility. Several contributing factors have now been identified. 1) Aggregation varies depending on emotional stress, recent exercise, and plasma lipid levels.<sup>10, 11</sup> 2) The aggregation of platelets in response to a fixed dose of adenosine diphosphate (ADP), for example, varies predictably depending on the age in minutes of the sample.<sup>12</sup> 3) With control of these variables, platelet aggregation will still vary significantly in a healthy man studied from one day to the next.<sup>13</sup>

We have attempted to control the above variables in a search for a possible acute effect of cigarette smoking on platelet aggregation. By varying the content of the cigarette smoked, in a double blind fashion, we have also studied whether cigarette smoke itself is responsible for the changes observed. Finally, we have observed the increase in plasma free fatty acids induced by smoking,<sup>14</sup> and studied its relationship to changes in platelet function.

#### **Materials and Methods**

Platelet aggregation studies were performed by the optical density method of Born,<sup>15</sup> as modified by Mustard et al.<sup>16</sup> Blood was collected in plastic syringes and immediately mixed (9:1) with Ware's anticoagulant (6 parts 0.1 M sodium citrate to 4 parts 0.1 M citric acid) in plastic tubes. The blood was centrifuged at 200 G for 6 min at 22°C and the platelet rich plasma

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(PRP) was removed. The remaining blood was then centrifuged at 850 G for 15 min to yield platelet poor plasma (PPP). The platelet count of the PRP was adjusted to  $200-300 \times 10^3$ /cu. mm by dilution with PPP. This material was placed in the cuvette of a Chrono-Log aggregometer and the light transmittence was recorded on a moving strip chart recorder. The blank for each study was a similarly treated sample of PPP. When the addition of ADP caused the isolated platelets to form aggregates, light transmittence increased proportional to the size of the aggregates.

Disodium ADP was dissolved in veronal buffer (pH 7.35) to a concentration of 10 mg % adjusted to pH 6.8, and frozen in aliquots at  $-40^{\circ}$ C. For each subject, a dose of ADP was chosen which would just produce a small reversible primary wave of aggregation, and this dose was then used throughout. The range in ADP concentration for this effect was between 1.3 and 2.6x10<sup>-6</sup>M. It was hoped that this relatively small ADP stimulus would unmask pre-existing qualitative platelet changes, and would also be closer to "physiologic" levels of aggregating stimuli.

Other parameters measured on each sample included: platelet count, platelet factor 3 release by the method of Zucker and Peterson,<sup>17</sup> and plasma free fatty acids (FFA) by the method of Dole.<sup>18</sup>

Twenty seven healthy male and female volunteers were requested to abstain from all medications for 10 days and from all caloric intake and cigarette smoking for at least 12 hours prior to study. Each subject was placed in a reclining chair, and a #19 butterfly-type needle inserted into the largest antecubital vein. A slow infusion of physiologic saline was begun to keep the needle patent. This avoided the stress of recurrent venepuncture. Following 30 to 60 min rest, blood samples were drawn every 10 min via the butterfly needle, using a two syringe technique. The first several blood samples were discarded, until the subject appeared to be "comfortable" with the protocol. The total blood drawn during the entire study was between 250 and 300 ml per patient.

After several baseline measurements were obtained, the subject smoked a single cigarette, and another series of studies were done at 10 min intervals. After a period of 30 to 60 min, a second cigarette was smoked and a third series of studies performed. One of the cigarettes was a standard commercial filtered brand, containing 1.3 mg nicotine. The other was a filtered cigarette in which lettuce leaf was substituted for tobacco;<sup>19</sup> these cigarettes have no nicotine content. To preclude observer bias, those performing and calculating the platelet aggregation studies were not told which samples were pre or post smoking or which cigarette had been used. To minimize artifacts due to ageing of platelet samples, all studies were performed in a rigid time-controlled manner, and were completed within a maximum of 30 min from time of blood drawing.

#### Results

Serial Studies. Serial measurements of platelet aggregation under "baseline" conditions showed excellent reproducibility. Variability in 40 sets of experiments never exceeded 10 percent above or below the mean in any subject. On three occasions, difficulties with blood return from the intravenous needle required repeat venepuncture. Following this procedure, there was a significant rise in aggregation response. For this reason, any patient who required a second venepuncture was excluded from the study.

Effect of Emotional Stress. Following baseline measurements, 10 healthy volunteers were requested to "smoke" a standard cigarette in the usual manner, without lighting it. During this maneuver, the investigators continuously reinforced the subject's awareness that he was being closely observed. The mean platelet aggregation response measured 10 and 20 min before such sham smoking was compared to the mean response 10 and 20 min afterwards. No significant difference was found. The results are shown in the first column of figure 1.



Maximum platelet aggregation in response to a fixed dose of ADP. Paired experiments before and after sham smoking, non-nicotine cigarette smoking, and standard cigarette smoking.

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Results in Non-Smokers. Platelet aggregation was measured before and after cigarette smoking in 6 non-smokers. All had significant difficulty in inhaling, with resultant paroxysms of coughing. This "stress reaction" precluded meaningful interpretation of data. Results were erratic. Of interest were the plasma FFA levels, which were not significantly increased by smoking in these subjects. This suggested that relatively little nicotine had been

Acute Effects in Smokers: Lettuce Leaf. The mean platelet aggregation responses measured 10 and 20 min before and after smoking the lettuce leaf cigarette were compared in 11 subjects. No statistically valid difference was noted. These results are shown in the second column of figure 1.

absorbed.

Acute Effects in Smokers: Tobacco. On the same day, similar studies were performed in the same 11 subjects before and after smoking the standard cigarette, under the same experimental conditions. Results are shown in the final column of figure 1. Using the *t*-test for paired data, the increased aggregation seen following standard smoking was compared to the change in aggregation from lettuce leaf cigarette smoking. The difference was significant at the P < .01 level. To determine whether prior exposure to lettuce leaf smoking had "sensitized" the patient to the second cigarette, the order of testing was reversed in 3 subjects. The results were unchanged; increased aggregation response followed the standard cigarette only.

Timing of Response. Serial measurements (fig. 2) showed no increase in aggregation immediately following cigarette smoking. Increase in aggregation was maximum at 10 and 20 min, and at or near baseline by 30 min. Two patterns were noted. The commonest (fig. 2, top line) was an increase in primary wave, with loss of complete disaggregation. Less often, the primary wave was of sufficient magnitude to result in a release reaction, with a secondary wave of aggregation resulting (fig. 2, bottom line).

Free Fatty Acids. None of the above subjects developed an increase in FFA after the lettuce leaf cigarette. After the standard cigarette, all eleven developed increased FFA. The mean rise was 134 mg% from a mean baseline level of 556 mg%. The time of maximal free fatty acid rise was 30 min after smoking. The degree of increment for individual subjects did not correlate with the degree of enhancement of aggregation. In three subjects, the rise in FFA occurred only after the increase in aggregation was noted.



Serial platelet aggregation curves before and after smoking a standard cigarette. Two typical subjects are shown. Free fatty acid concentration in milli-equivalents per liter of plasma, as shown by dashed line.

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*Platelet Factor* 3. PF3 measurements did not differ significantly in any of the groups studied.

#### Discussion

The smoking of a single cigarette results in some qualitative change in the platelet which renders it more responsive to exposure to low doses of ADP. This does not appear to be due to the stress of the protocol, and it does not occur following the smoking of nicotine-free cigarettes. Nicotine causes a rise in plasma epinephrine levels,<sup>20</sup> and this has been shown to occur in cigarette smokers. The rise in free fatty acids is thought to be a result of the epinephrine increase.<sup>21</sup> The timing and magnitude of the increase in FFA observed by us is in agreement with the previous data of Kershbaum et al.<sup>22</sup> We had expected that the free fatty acids would in turn be responsible for the observed increase in platelet aggregability, but our data did not suggest this.

The enhancement of platelet aggregation observed after smoking could be a direct effect of nicotine, or could be due to the known increase in plasma catecholamines.<sup>20</sup> (We were unable to measure plasma epinephrine levels in parallel with platelet function in this study, because of the large volumes of blood which such determinations would have required.) Catecholamines may play a major role in the *in vivo* platelet aggregation response. Epinephrine is a well-studied stimulus of platelet aggregation and the release reaction.<sup>23, 24</sup> Moreover, the addition of epinephrine to platelets *in vivo* has recently been shown to enhance their subsequent response to ADP-induced aggregation.<sup>25</sup>

Cigarette smokers have an increased risk of myocardial infarction<sup>26</sup> as well as sudden death.<sup>2</sup> In experimental animals also, *in vivo* induction of platelet aggregates can lead to both myocardial infarction and sudden death.<sup>27</sup> If a single cigarette can sensitize blood platelets to ADP, such platelets would hypothetically be more ready to participate in platelet plug formation, leading to vascular occlusion.

The data presented here suggest a possible direct causative link between cigarette smoking and arterial thrombotic disease. Moreoever, there is literature<sup>28–31</sup> which suggests that the encrustation of layers of platelets on the arterial wall, at sites of turbulent blood flow and/or endothelial damage, may be the precursor of the atherosclerotic plaque. If this literature has any validity, the results of the present study may also help to explain the mechanism by which smoking may accelerate atherosclerosis. These considerations lend added importance to the development and study of agents which can effectively modify platelet reactions.

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