EFFECT OF SMOKING ON BLOOD COAGULATION AND PLATELET SURVIVAL IN MAN*

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The role of smoking in the production of vascular disease and its complications has attracted attention. It has been heavily incriminated in Buerger's disease (Allen *et al.*, 1955), and is apparently associated with some increased incidence of coronary artery disease (Doll and Hill, 1956; Hammond and Horn, 1958; Doyle *et al.*, 1961). It is not clear by what mechanism or mechanisms these processes are related. Kershbaum *et al.* (1961) have found that smoking produces a rise in the plasma free fatty acid; the effects of smoking on the blood-pressure and cardiovascular dynamics have been extensively documented; and there is evidence that smoking influences blood coagulation (Horwitz and Waldorf, 1960). The studies of Shimamoto and associates (personal communication) in rabbits on the effects of inhaling cigarette smoke on the adhesion of platelets to the endothelium in the microcirculation are of particular interest. They have found that the endothelium cells are structurally altered in the process.

There are considerable technical difficulties in the use of *in vitro* tests for the study of blood coagulation and platelet economy, and some investigators have had misgivings about the interpretation of results obtained under such circumstances (O'Brien, 1957). It has been felt that *in vivo* studies might correspond more closely to reality. The best quantitative technique of this kind at present available is the measurement of platelet survival, and this method is all the more valuable in view of the well-established importance and primary role of the platelet in the production of arterial thrombi (Best *et al.*, 1938; Poole and French, 1961). It seems probable that the fate of the circulating platelet depends on three factors—ageing of the platelet, deposition on the vascular endothelium, and participation in blood coagulation (Cronkite *et al.*, 1961). If, indeed, smoking affects the arterial wall or endothelium, or blood coagulation, we might reasonably expect this to be reflected in the survival of the blood platelets.

The greater part of the evidence indicates that diet influences *in vitro* tests of blood coagulation (Mustard, 1961), and we have recently shown that this is also true of platelet survival (Mustard and Murphy, 1962). It is therefore clearly important that studies such as the one reported in this paper should be done under metabolic ward conditions, especially in view of the relationship between change of smoking habit and appetite.

Accordingly, we have studied blood coagulation and platelet survival in seven white male subjects, each of them during two periods, one in which they were allowed to smoke and one in which they were not.

Methods

Subjects.—A varied group of seven white male veterans was studied. Their ages (given in Table II) ranged between 35 and 72. Three had sustained myocardial infarctions, two cerebrovascular accidents, and one had had recurrent thrombophlebitis, but there had been no overt episodes of vascular disease in any of the patients for some months before the experiment was done. The seventh subject had chronic bronchitis and emphysema without evidence of vascular disease. All were moderately heavy smokers (at least 20 cigarettes a day), but all agreed to refrain from smoking for three weeks immediately prior to one test as well as during the 10 days during which the test was done. Although we cannot be absolutely certain that none of them lapsed occasionally, we are at least certain that their cigarette consumptions were greatly reduced.

Diet.—A diet of uniform composition and caloric content was maintained throughout. The weight of each subject was maintained constant to within 2 lb. (0.9 kg.). The diets had the following composition: fat 22% of calories, protein 15%, carbohydrate 63%. The diets contained no dairy fat or egg yolk except for 7 oz. (200 ml.) of skim milk daily; 30% of the fat was derived from vegetable sources.

Experimental Design.—This was a simple crossover design, three of the subjects selected at random being studied during the non-smoking period first and the smoking period second, and the remaining four in the reverse order. Since the same subjects were studied in the two periods no problem of comparability arises, and comparisons can be made by pairing the results.

Coagulation Tests.—These comprised the whole-blood clotting-time, the one-stage prothrombin time, the plasma thromboplastin time, the platelet clumping-time, the platelet adhesive index, and the platelet count. The techniques involved have already been described in detail (Murphy and Mustard, 1960). Samples of blood were taken daily during the 10-day period in which platelet survival was studied. All were taken in the morning with the patient fasting overnight.

Platelet Survival

This was measured by a modified form of the diisopropyl fluorophosphonate ^{32}P (D.F. ^{32}P)* method of Leeksma and Cohen (1956), which we have previously described (Murphy and Mustard, 1961a). In this method platelets are tagged *in vivo* by this tenacious radioactive compound. We have in the last two years explored and introduced further modifications which we have found to improve the precision of the method. Each blood specimen is taken into 3.5 ml. of ethylenediaminetetra acetate solution containing 1 ml. of dextran† per 20 ml. of blood in a silicone-coated glass tube. The blood is then allowed to stand for two to three hours at room temperature. After this time the supernatant platelet-

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†Dextran 6% and NaCl 0.9% ("intradex").

rich plasma is transferred to a silicone-coated glass centrifuge tube and centrifuged at 1,000 r.p.m. (R.C.F. 225) at 4° C. in an M.S.E. major refrigerated centrifuge for 12 minutes. The supernatant platelet-rich plasma is then centrifuged at 2,600 r.p.m. (R.C.F. 1,540) for 15 minutes at room temperature. The resulting platelet button is then washed twice with saline as described previously. The part of the platelet button eventually put in the planchette is essentially free of red blood cells (less than one red cell per 25,000 platelets) and white cells (less than one white cell per 40,000 platelets).

Instead of enumerating the platelets in the final suspension before drying, we now weigh the dried deposit. This makes it necessary that the final suspension should be in distilled water, not in saline as previously described. Only the upper half of the platelet button is suspended in distilled water. The methods and problems of computing platelet survival from the data have been discussed elsewhere (Murphy and Mustard 1961a, 1961b, Mustard and Murphy, 1962). There is controversy about the pattern which platelet survival follows. Obviously, there are many possible patterns, but the two which have proved most attractive are:

1. That platelets are indiscriminately destroyed. Under this model, arguing deterministically, one would expect an exponential decay curve.

2. That platelets have a definite life-span which follows a Gaussian distribution. The details of the decay curve would then depend on the nature of the population of platelets labelled—for example, this would be different for a cohort label from what would be expected with a total population—and on the coefficient of variation. For total population labelling such as is achieved by the intravenous injection of D.F.^{32}P., the actual survival curve would be a straight line if the standard deviation of the curve is indefinitely small. This has been widely referred to in the literature as a "linear" decay, although it is evident that when the standard deviation of cell survival is considerable the decay is not linear.

It is probable that both mechanisms operate, but as yet no method has been devised for determining which is the more important. Accordingly, here as elsewhere, we compute survival under both mathematical models. We have evidence from animal experiments that random destruction is probably the more important mechanism (Robinson, Murphy, Rowsell, and Mustard, unpublished observation), and we may note that the platelet half-life computed on the exponential model gives at once the better discrimination between groups and the better reproducibility (see below).

Experimental Error

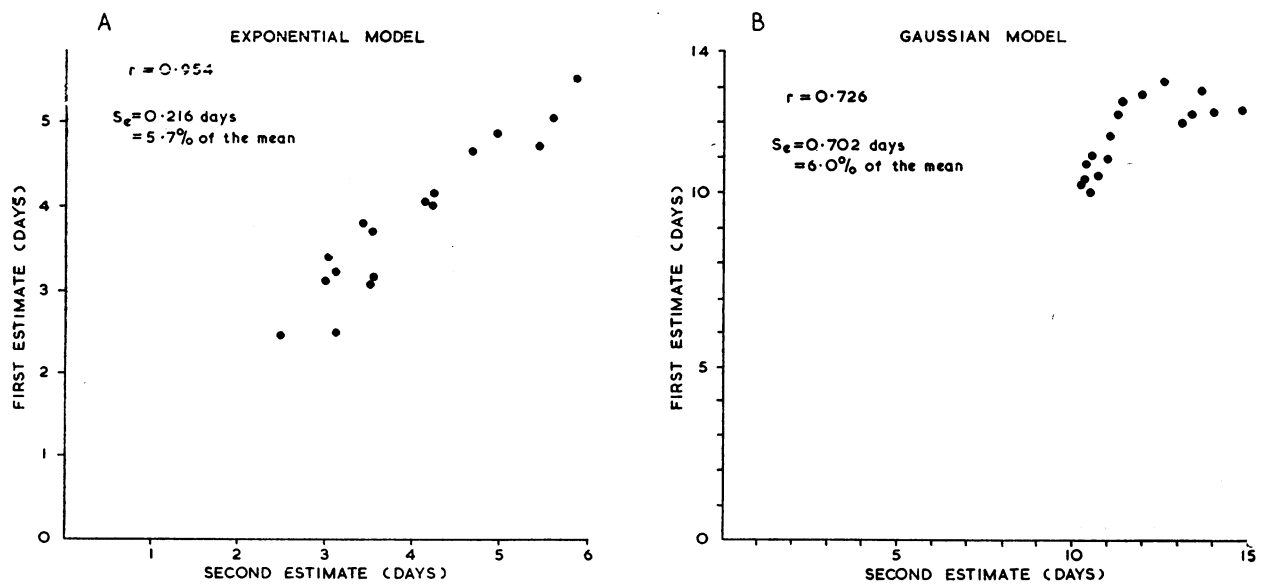
In using these tests it is important that we should have some idea of their precision. This has been explored by the same techniques in a group of 17 subjects—some in-patients, some out-patients—distinct from the subjects of the present paper. The results of duplicate studies at variable times of the year are shown in Table I and the Chart. The reproducibility of the methods is represented in various ways. (1) Correlation coefficients, which are perhaps the most informative. This is found to be excellent when survival is computed on the exponential model. (2) Standard experimental errors, which make it easy to compare error from this source with differences in mean values encountered between various groups. (3) Percentage experimental error, which conforms to general usage.

TABLE I.—*Reproducibility Studies on In-vivo Measures of Platelet Economy**

Measure	Correlation Coefficients between First and Second Studies	Standard Experimental Error	Percentage Error
Platelet count (No./c.mm.)	0·689	43,100	19·33
Exponential platelet half-life (days)	0·954	0·216	5·69
Exponential platelet turnover (No./c.mm./day)	0·782	8,302	19·21
Gaussian mean survival (days)	0·726	0·702	5·98
Gaussian platelet turnover (No./c.mm./day)	0·639	4,184	21·98

* Based on 17 pairs of readings.

The tests were done under conditions which were not rigorously controlled but which were fairly constant for any one subject. Differences among subjects were in part due to the differences of the conditions under



Comparison between the estimate of platelet survival in the first and second tests. A: Under the assumption of random destruction. B: Under the assumption that platelet survival is normally distributed.

which they were maintained, in part to the nature of the patient's disease.

Statistical Considerations.—We have shown elsewhere that the whole-blood clotting-time and the platelet clumping-time are log-normally distributed, the plasma thromboplastin tissue has a harmonic normal distribution, and the other variates are normally distributed (Murphy and Mustard, 1961a, 1961b). Appropriate transformations have been used in computing means and differences between groups, but mean values have been retransformed to the original scale before being entered in the tables.

Results

In-vitro Tests of Coagulation.—The means of several results for each test on each subject are given in Table II. The platelet clumping-time is prolonged by 20% when the subjects cease smoking. This is just significant at the 5% level but is an almost uniform change, subject 6 being the only exception. Among the other four tests performed there are no significant differences between the two groups.

In-vivo Tests.—The results are given in Table III. On the exponential model there is a prolongation of platelet survival of nearly a day. This trend is uniform for all seven subjects. The difference is highly significant and the mean difference is almost four times the standard experimental error given in Table I. Platelet turnover based on these figures shows corresponding changes—that is, a decrease in the non-smoking figure, and this too is uniform. For survival and turnover computed on the Gaussian model the differences are similar but not quite so marked. The mean platelet counts are almost identical for the two groups.

Serum Lipids.—The mean serum cholesterol and phospholipid values for each subject during each of the two periods of study were similar. The differences between the mean values for the two periods were not significant (Table IV).

TABLE IV—*Effect of Smoking on Various Blood Lipids*

Subject	Mean Serum Cholesterol (mg. 100 ml. Blood)		Mean Serum Cholesterol Ester (mg. 100 ml. Blood)		Mean Serum Phospholipid (mg. 100 ml. Blood)	
	Smoking	Non-smoking	Smoking	Non-smoking	Smoking	Non-smoking
1	264 (6)	276 (5)	193 (6)	204 (5)	233 (6)	228 (5)
2	218 (2)	213 (4)	164 (2)	170 (4)	209 (2)	216 (4)
3	235 (6)	217 (6)	181 (6)	167 (6)	197 (6)	200 (6)
4	264 (4)	242 (3)	194 (4)	184 (3)	234 (4)	221 (3)
5	203 (4)	187 (5)	151 (4)	144 (5)	183 (4)	170 (5)
6	174 (4)	182 (2)	124 (4)	132 (2)	179 (4)	186 (2)
7	146 (6)	160 (5)	108 (6)	122 (5)	172 (6)	186 (5)
Mean of means	215	211	159	160	201	201
t (paired differences)	0·67		0·27		0·00	
P	<0·6		<0·9		=1·0	

Note: The numbers in parentheses after each average indicate the number of estimates on which that average is based.

Discussion

Even in this small group of patients it is clearly demonstrable that platelet survival is shortened by smoking and that this is associated with an increased turnover of platelets. It is not clear through what mechanism these changes are mediated. Three possibilities may be considered: (1) that smoking affects the platelet directly; (2) that smoking accelerates blood coagulation and that the platelet is secondarily involved; and (3) that smoking changes the physical properties

TABLE II.—*In-vitro Coagulation Tests (Mean Values)*

Subject	Age	Diagnosis	Whole-blood Clotting-time (Minutes)		Prothrombin Time (Seconds)		Plasma Thromboplastin Time (Seconds)		Platelet Clumping Time (Seconds)		Platelet Adhesive Index	
			Smoking	Non-smoking	Smoking	Non-smoking	Smoking	Non-smoking	Smoking	Non-smoking	Smoking	Non-smoking
1	42	C.D.	12·2	10·8	15·1	16·0	12·45	11·83	309	406	1·17	1·32
2	35	R.T.	9·7	10·9	15·9	14·9	11·94	13·63	258	317	1·11	1·10
3	48	C.D.	11·0	12·0	15·2	14·3	12·40	10·88	206	352	1·24	1·36
4	65	C.V.D.	11·9	12·6	15·6	16·0	12·67	12·12	272	301	1·05	1·20
5	59	C.V.D.	11·8	9·4	15·8	15·5	13·25	12·05	243	270	1·07	0·99
6	72	C.B.E.	8·6	10·8	16·0	15·7	13·49	12·16	291	270	1·18	1·25
7	60	C.D.	10·4	12·6	17·1	16·2	11·78	12·52	281	316	1·24	1·09
Mean of means			10·7	11·3	15·82	15·50	12·54	12·12	264	316	1·15	1·18
Significance of differences between means: t			0·82		1·138		0·94		2·52		0·77	
P			<0·5		<0·3		<0·4		<0·05		<0·5	

C.D.=Coronary disease. R.T.=Recurrent thrombophlebitis. C.V.D.=Cerebrovascular disease. C.B.E.=Chronic bronchitis and emphysema.

TABLE III.—*Platelet Survival and Turnover Values*

Subject	Platelet Count (No./c.mm. × 1,000)		Platelet Half-life Exponential (Days)		Platelet Turnover Exponential (No./c.mm. day × 10^3)		Platelet Survival Gaussian (Days)		Platelet Turnover Gaussian (No./c.mm./day × 10^3)	
	Smoking	Non-smoking	Smoking	Non-smoking	Smoking	Non-smoking	Smoking	Non-smoking	Smoking	Non-smoking
1	231	241	2·99	3·27	53·20	51·10	10·62	10·87	21·76	22·15
2	174	212	4·27	5·43	28·27	27·12	12·11	13·86	14·37	15·33
3	353	389	3·61	4·96	67·70	54·30	11·61	12·93	30·40	30·00
4	236	250	4·67	5·79	34·96	29·92	12·25	14·02	19·25	17·83
5	215	230	5·25	6·54	28·31	24·37	12·90	14·85	16·67	15·43
6	347	255	3·99	4·11	60·21	43·02	11·95	11·95	29·00	21·34
7	287	242	3·49	4·20	57·20	39·90	11·26	11·26	25·56	21·51
Mean	263·2*	259·9*	4·038	4·900	47·122	38·533	11·810	12·819	22·430	20·522
Significance of differences between means: t	0·19		4·55		3·19		2·99		1·68	
P	<0·9		<0·005		<0·02		<0·05		<0·2	

* Means of means.

of the arterial wall and its endothelium and that platelet and perhaps coagulation changes are secondary.

There is little evidence relating to the effect of smoking directly on the platelet. It is of interest in this study that platelet clumping *in vitro* was accelerated by smoking, but this was only just significant, and it is possible that it is an artifact which has arisen as a result of the multiplicity of the comparisons. Certainly it gains no confirmation from the studies on platelet adhesive index. We know of no supporting evidence in the literature, and further investigation will be necessary to confirm or reject it.

As to the second point, there is evidence from acute experiments using the thromboelastograph that smoking a cigarette shortens the time required for the development of the maximum tension of the clot (Horwitz and Waldorf, 1960); this probably corresponds to a change in the rate of clot formation, but not to the time of onset of clotting. This present study focused not on the acute effects of smoking a single cigarette but rather on the accumulated consequences of sustained periods with and without intermittent smoking. It is thus possible that, since we did not do our studies routinely after the patient had smoked a cigarette, we may have failed to detect transient changes in coagulation. However, an alternative explanation for the sequence of events in the acute experiments is that the changes in coagulation were secondary to changes in the platelet.

The third possibility is that the primary effect of smoking is on the arterial wall and its endothelium. Shimamoto's experiments suggest that such a mechanism may indeed be important. The evidence from our study is compatible with the explanation that the endothelium is damaged and that there is increased adhesion of platelets at this time. On such a hypothesis one might expect that coagulation changes would be trivial. However, it may be pointed out that our studies were done on a small group of subjects and the tests themselves have a large experimental error, so that it would be unwise to attempt to come to any firm negative conclusions on this point.

There were no significant changes in serum-cholesterol levels during the period of study. Although this evidence suggests that smoking has little effect on these lipid measurements (perhaps because the subjects were maintained on an even dietary intake throughout the two periods), it provides no evidence about changes in fatty acids. Fatty acids of certain kinds enhance thrombus formation (Connor and Poole, 1961) and can influence blood coagulation (Poole, 1958). It has been shown that smoking increases the fatty-acid content of plasma (Kershbaum *et al.*, 1961). In acute experiments smoking was not found to increase the serum-cholesterol level (Page *et al.* 1959). This evidence, together with that from the present study, may indicate that the higher mean serum cholesterol reported in smokers as compared with non-smokers (Thomas, 1960; Spain and Nathan, 1961) is not due to smoking; perhaps they should be attributed to differences in dietary habit, or perhaps hypercholesterolaemia and the smoking habit are independent consequences of some common cause. Konttinen (1962), however, in an extensive investigation in young men found no differences in serum cholesterol and serum phospholipid in smokers and non-smokers in Finland.

These considerations by no means exhaust the possibilities about the means by which smoking influences platelet survival. For example, psychomotor activity is one of the factors which we have not been able to control. We have no evidence bearing on this or on what effect (if any) this may have on platelet economy.

It has been difficult to decide whether cigarette smoking is causally related to the development of coronary atherosclerosis and its complications or whether it is simply one of the characteristics of certain individuals who possess other factors which are causally related. In view of the primary role of platelets in arterial thrombosis, it is possible that the changes in platelet economy found in the present study are an indication that smoking may influence thrombosis. Since thrombi, and therefore platelets, contribute to the development of atherosclerosis (Duguid, 1948; More and Haust, 1961; Mustard *et al.*, 1961, 1963) it is conceivable that the changes found in the study mean that smoking can influence the development of atherosclerosis as well as its complications. However, much more work will have to be done before this point is settled.

Summary

In paired studies on seven white male subjects under metabolic ward conditions, platelet survival was significantly shorter and platelet turnover correspondingly greater when the subjects were habitually smoking than when they were not smoking at all. This was associated with only minor changes in blood coagulation or blood-lipid levels.

We think that this may have an important bearing on the relationship between smoking and vascular disease.

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