Comparison of Leukocyte Count and Function in Smoking and Nonsmoking Young.Men

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Leukocyte function and other hematological measurements were tested in 14 smoking and 13 nonsmoking young men free of intercurrent or chronic disease. Leukocyte chemotaxis was depressed in smoking subjects when compared to the same subjects abstaining from cigarettes or to the nonsmokers. Smoking did not affect the whole blood bactericidal capacity of leukocytes and serum for *Staphyloccus aureus* or *Klebsiella pneumoniae*. Total leukocyte counts, hematocrits, and monocyte counts were higher in the smoking subjects when compared to the nonsmokers.

Although pulmonary macrophages in smoking and nonsmoking individuals have been the subject of numerous studies, relatively little attention has been focused on the peripheral blood leukocytes of cigarette smokers. The processes of leukocyte chemotaxis and phagocytosis are complex and are affected by a large number of diverse conditions and drugs (25). The present study was designed to test the effects of acute and chronic cigarette smoking on the leukocytes of healthy young men. Because of the possibility that a rapidly labile leukotoxic agent may exist in the blood after cigarette smoking, emphasis was placed on tests which rapidly measure leukocyte function such as the whole-blood phagocytic and bactericidal test described by Castro et al (6). In this test, separation or manipulation of the leukocytes is not performed. A *Staphylococcus* and a *Klebsiella* were chosen as test organisms because the former is killed primarily by phagocytic mechanisms and the latter predominantly by the serum bactericidal reaction (23). Leukocytes from smoking and nonsmoking men were also tested by a modification of the Boyden method (4) for their ability to respond to a chemotactic stimulus.

MATERIALS AND METHODS

Subjects. The participants in the study were white, male medical students and house officers from 20 to 30 years of age. Three groups were selected: nonsmokers, abstaining smokers, and smoking smokers. The first group, nonsmokers, included 13 men. The smoking subjects were 14 men who currently smoked at least 20 cigarettes per day. These individuals were tested twice to make up the final two groups. Smoking individuals were tested once following overnight abstention from smoking (abstaining smokers) and again immediately after smoking two unfiltered cigarettes,

each containing approximately 0.89 g of tobacco (25 mg of tar, 1.6 mg of nicotine/cigarette, U.S. Federal Trade Commission Report, August 1973). The latter group (smoking smokers) smoked the two cigarettes consecutively. Subjects were not accepted if they gave a history of diabetes mellitus, asthma, chronic bronchitis, splenectomy, sickle cell disease, intercurrent or recent (past week) infectious illness, or recent (24 h) ingestion of alcohol, nose drops or sprays, tranquilizers, prednisone, aspirin, or antibiotics including isoniazid. The subjects were advised to refrain from strenuous exercise prior to the blood donation, which was performed by venipuncture between 9 and 10 a.m. The participation of the subjects in this study was approved by the Committee on Human Investigation and Study of the University of Kentucky Medical Center.

Hematological studies. Leukocyte counts were done in a hematocytometer. Differential counts were performed by counting 100 cells from a Wright-stained smear. The differential counts were done by one technician who did not know which subject was being tested. Hematocrits were performed in a capillary tube (1.1 to 1.2 by 75 mm).

Microorganisms. The bacteria used in the studies of phagocytosis were *Staphylococcus aureus* 12600-2, serotype 3, and *Klebsiella pneumoniae* 8052, type 2 (American Type Culture Collection, Rockville, Md.). Each organism was grown for 18 h at 37 C in tryptic soy broth (Difco, Detroit, Mich.) with added 7.5% glucose, and aliquots of the cultures were lyophilized in small vials for storage. Fresh isolates of the bacteria were prepared from the lyophilized stocks at weekly intervals, and inocula utilized for bactericidal assays were obtained daily from blood agar or tryptic soy agar plates kept at 4 C.

Whole-blood phagocytosis and bactericidal activity. The method used was from that of Castro et al. (6), modified as follows. Dispersion of bacteria in liquid media (tryptic soy broth) was accomplished by means of vigorous pipetting and agitation with a mechanical shaker rather than by sonic treatment. The concentration of the bacteria was adjusted by

means of optical density to yield approximately 10^4 colony-forming units/ml of tryptic soy broth.. The suspension of bacteria (0.1 ml) was added to 0.9 ml of fresh defibrinated blood in a test tube (10 by 75 mm) and was sealed with a paraffin-covered cork stopper. The tube was rotated end over end at 8 rpm at 37 C. At times 0, 10, 30, and 60 min, 0.1-ml aliquots were withdrawn and plated directly into agar. The bacterial colonies in the agar plates were counted after 24 h of growth at 37 C. The number of bacterial colonies was expressed as a percentage of the number of bacteria that was found at zero time. The test was always initiated within 30 min of drawing the blood.

Leukocyte chemotaxis. The measurement of leukocyte chemotaxis was modified from that of Baum et al. (1). Blood (10 ml) was taken in a Vacutainer tube (Becton-Dickinson Co., Rutherford, N.J.) containing 143 USP units of heparin. To this was mixed 0.75 ml of a solution of 6% dextran in saline, and the erythrocytes were sedimented for 40 min. Leukocytes were removed from the plasma fraction and adjusted to approximately 7×10^6/ml with Gey medium containing 2% bovine serum albumin and sodium bicarbonate (Flow Laboratories, Rockville, Md.). Approximately 2.1×10^6 leukocytes containing between 65 and 75% neutrophils were deposited on a 3-μm membrane filter (Millipore Corp., Bedford, Mass.) by means of a Cytocentrifuge (Shandon Scientific Co., Sewickley, Pa.). The filters were then placed in a modified Sykes-Moore chemotaxis chamber (Bellco Glass, Inc., Vineland, N.J.). The starting side of the chamber was filled with Gey medium with 2% bovine serum albumin, and the attractant side was filled with a 1:1 dilution of Gey medium and activated serum (7). Activated serum was present in a concentration of 2.5% in the attractant side. An AB+ donor provided the serum, which was stored at −70 C prior to activation. When required, the serum was thawed on ice, and 0.05 ml was mixed with 0.10 ml of saline containing 30 μg of lipopolysaccharide B, *Escherichia coli* O127:B8, endotoxin (Difco), and 0.85 ml of dextrose gelatin veronal buffer, pH 7.2, with Ca^{2+} and Mg^{2+} (GIBCO, Grand Island, N.Y.). This mixture was incubated at 37 C for 1 h in a water bath, and the residual complement was inactivated by heating at 56 C for 30 min. Two test chambers were used for a single blood sample. The control chambers contained Gey medium with 2% bovine serum albumin on both sides of the filter. The chambers were incubated at 37 C for 1 h in an incubator, and the filters were removed and stained with Ehrlich hematoxylin (4). The filters were mounted in Permount (Fisher Scientific Co., Cincinnati, Ohio) with the attractant side down. The cells were observed with a Wild M20 microscope using the ×40 objective and ×10 ocular. The distance migrated by the cells was measured between the cells on the starting side of the filter and the most distant two cells simultaneously in focus on the attractant side (28). The measurement was performed with the micrometer on the fine focus of the microscope. The measurements were performed five times per filter and were reported as an average of duplicate filters. Although not done with each experiment, random movement as a cause for the chemotaxis results was ruled out by a series of tests placing

activated serum on both sides of the filter. Unless otherwise stated, the *t* test for paired samples and the *t* test for two independent samples were used to compare each smoking group with the nonsmoking group.

RESULTS

Hematological studies. In Table 1, the hematocrits of the smoking smokers are seen to be the highest of the three groups. This difference was significant ($P < 0.05$) in comparison to the nonsmokers. When the hematocrits of the smoking smokers were compared to those measured in the same individuals on the day on which no smoking took place, a P value of 0.08 resulted. The total leukocyte counts were significantly higher in both the smoking ($P < 0.001$) and abstaining smokers ($P < 0.01$) when compared to the nonsmokers. Table 2 is a comparison of the average percentages of leukocytes by type in the three groups. The percentage of neutrophils was lowest in the abstaining smokers in comparison to the smoking smokers ($P < 0.02$) and the nonsmokers ($P < 0.02$). However, the abstaining smokers had the highest percentage of lymphocytes in comparison to the smoking smokers ($P < 0.04$) and the nonsmokers ($P < 0.15$). Further, the smoking groups had higher percentages of monocytes than did the nonsmoking group, the abstaining smokers significantly so ($P < 0.02$). The differences among the basophils and eosinophils were not subjected to statistical analysis because of the small numbers involved in each group. Table 3 demonstrates comparisons between the absolute numbers of leukocytes by types in the three groups. The number of neutrophils was significantly higher ($P < 0.03$) in the smoking smokers than in the same individuals abstaining from cigarettes. The mean neutrophil counts in the abstaining smokers were similar to those in the nonsmoking group. The number of lymphocytes was highest in the abstaining smokers and

TABLE 1. *Comparison of the means of hematocrits and numbers of leukocytes in smokers and nonsmokers*

Group[a]	Hematocrit	Leukocytes/mm³
SS (14)	47.1%	7,625
AS (13)	46.6%	7,485
NSm (13)	45.0%	5,934
SS vs. AS	NS[b]	NS
SS vs. NSm	$P < 0.05$	$P < 0.001$
AS vs. NSm	NS	$P < 0.01$

[a] Abbreviations: SS, Smoking smokers; AS, abstaining smokers; NSm, nonsmokers.

[b] NS, Not significant at the 0.05 level.

TABLE 2. Comparison of mean percentage of leukocytes by type in smokers and nonsmokers

Group[a]	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
SS (11)	55.1	2.64	1.73	33.1	7.73
AS (11)	48.5	2.82	.36	39.6	8.55
NSm (12)	56.4	2.17	.50	35.1	5.75
SS vs. AS	$P < 0.02$			$P < 0.04$	NS[b]
SS vs. NSm	$P < 0.02$			NS	NS
AS vs. NSm	NS			NS	$P < 0.02$

[a] Abbreviations as in Table 1.
[b] NS, Not significant at the 0.05 level.

TABLE 3. Comparison of mean number of leukocytes by type in smokers and nonsmokers

Group[a]	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
SS (11)	4,123	97	196	2,412	565
AS (11)	3,527	22	230	2,846	591
NSm (12)	3,538	28	156	2,132	359
SS vs. AS	$P < 0.03$			NS[b]	NS
SS vs. NSm	NS			NS	$P < 0.01$
AS vs. NSm	NS			$P < 0.01$	$P < 0.01$

[a] Abbreviations as in Table 1.
[b] NS, Not significant at the 0.05 level.

significantly different ($P < 0.01$) from the number in the nonsmoking group. The monocyte counts in both smoking and abstaining smokers were significantly higher ($P < 0.01$) than those in the nonsmoking group.

Leukocyte phagocytosis and bactericidal activity. Tables 4 and 5 show the results of the phagocytic and bactericidal assays on whole blood among the three groups. Linear regressions performed on the killing curves showed no significant differences for the slope or the intercept of the three groups. However, we found that the mean zero time counts of *Klebsiella* and *Staphylococcus* in the smokers and abstaining smokers were significantly lower than the counts in the nonsmoking group, with P values in all comparisons <0.05. This, along with the elevated leukocyte count in the smoking groups, resulted in doubling the leukocyte/bacteria ratio in the smokers versus the nonsmokers.

To ensure that the unanticipated differences in the leukocyte/bacteria ratios did not mask an effect of smoking, additional experiments were performed. On 6 separate days, blood from a nonsmoker was inoculated with two quantities of each test organism to test the effect on the killing curves of altering the leukocyte/bacteria ratios. No effect was detected on the two killing curves for *Klebsiella* when the leukocyte/*Klebsiella* ratios were altered from 743:1 to 477:1 ($P < 0.025$). When the leukocyte/

Staphylococcus ratios were altered from 820:1 to 450:1, one point on the killing curve differed significantly between the two groups. This occurred at 30 min, when the percentages of the remaining organisms were 53.4 and 65.7 ($P < 0.025$), respectively. No significant difference occurred among the remaining points on the killing curves.

Leukocyte chemotaxis. The chemotaxis data are shown in Table 6. Leukocyte chemotaxis was present in each of the groups studied as evidenced by the fact that the difference between the test and control value for each group was significant ($P < 0.01$) using the one-sample t test. The difference in distance moved by the leukocytes was significantly less in the smoking smokers when compared to the abstaining smokers ($P < 0.03$) and nonsmokers ($P < 0.01$). The variability of both test and control results was much greater in the smoking and abstaining smokers when compared to the nonsmoking group. The variances of the test and control values in the smoking group were significantly higher ($P < 0.01$) than the nonsmoking group when tested by the F test.

DISCUSSION

The elevated leukocyte counts found in smoking men in our study is in agreement with the findings of other investigators (5, 9, 13, 20, 21, 24). There are, however, only a limited number of studies which have examined the differential

leukocyte counts in smokers and nonsmokers. Corre et al. (9) found increased absolute numbers of neutrophils, lymphocytes, and monocytes in cigarette smokers when they were compared to a noninhaling or nonsmoking control group. In addition, Corre et al. and others have also examined the percentage counts of leukocyte types in smokers and found increases in the percentage of neutrophils and decreases in the percentage of lymphocytes (5, 9). Our study differs somewhat from the previous reports in that we tested the smoking population both while smoking and while abstaining from cigarettes. It is unclear in many of the studies when the leukocyte counts were performed in relation to the act of smoking. Also in contrast to others, our study included only healthy, white males in a narrow age range (20 to 30 years) who had no evidence of bronchitis or other infectious process. Thus, it is unlikely that the differences in the differential counts could be attributed to concurrent infection despite the fact that other studies indicate that even young cigarette smokers are more subject to respiratory infections than are nonsmokers (12). The neutrophilia and lymphophenia present in the smokers of the present and other studies are similar to the leukocyte differential of individuals subjected to stress. We were unable to find studies in which the leukocyte counts were investigated in relation to the administration of nicotine, a tobacco constituent which in small doses evokes a discharge of catecholamines from the adrenal medulla (27). The peripheral blood monocyte is thought to be the precursor of the pulmonary macrophage, and the latter cells are found in larger numbers in the lungs of smokers than in nonsmokers (15,

22). Thus, the elevated percentage and numbers of monocytes in the smoking population may reflect an increased production of these cells in smokers.

Eisen and Hammond described acute rises in the hematocrit caused by smoking (11). On the days that our smoking men abstained from cigarettes, their mean hematocrits were lower than when tested after smoking. Although this difference was not significant, the hematocrits of the smoking men were significantly higher ($P < 0.05$) than those of the nonsmokers. Other studies have found a positive correlation between cigarette consumption and packed cell volume or hemaglobin values (16, 19). One explanation for the apparent acute effect on the hematocrit is that smoking results in an increase in mean corpuscular volume (20) and, indeed, Eisen and Hammond found that the hematocrits dropped in subjects who refrained from smoking for a few days (11).

Cigarette smoking had no effect upon the whole-blood bactericidal and phagocytic tests with either the *Staphylococcus* or *Klebsiella* test organisms. It is unlikely that the differences among the leukocyte/bacterial ratios resulted in masking an effect caused by cigarette smoking. Castro et al. have found that normal leukocyte variations which produced leukocyte/bacterial ratios similar to ours do not appear to be a determining factor in the reduction of bacterial counts of the *Staphylococcus* (6). Our separate study of this problem indicates that similar alterations of the leukocyte/bacterial ratios do not affect the reduction of bacterial counts of *Klebsiella*. The difference found in the staphylococcal killing curves at 30 min when the leukocyte/bacterial ratios were altered is

TABLE 4. *Comparison of whole-blood phagocytosis and killing of Klebsiella in smokers and nonsmokers*

Group	Mean leukocyte/ bacteria ratio	Mean CFU[a] at 0 time	Mean % CFU remaining at:		
			10 min	30 min	60 min
Smoking smokers (14)	753	1.01×10^4	18.1	0.6	0.2
Abstaining smokers (13)	604	1.24×10^4	24.7	1.6	1.1
Nonsmokers (12)	347	1.71×10^4	23.8	4.5	0.8

[a] CFU, Colony-forming units.

TABLE 5. *Comparison of whole-blood phagocytosis and killing of Staphylococcus in smokers and nonsmokers*

Group	Mean leukocyte/ bacteria ratio	Mean CFU[a] at 0 time	Mean % CFU remaining at:		
			10 min	30 min	60 min
Smoking smokers (14)	639	1.19×10^4	73.9	49.8	23.4
Abstaining smokers (13)	616	1.20×10^4	84.3	59.5	30.6
Nonsmokers (12)	373	1.59×10^4	78.2	56.0	24.9

[a] CFU, Colony-forming units.

not likely to be present if bloods from more than one individual were tested. Thus, the absence of an effect of tobacco smoking on phagocytosis as measured by our study of the human leukocyte extends the observations of Harris et al. and others that phagocytosis and killing of bacteria by human alveolar macrophages is normal in smokers (8, 15). It is possible that other methods of measuring leukocyte phagocytosis may demonstrate adverse effects of tobacco smoking. However, adverse effects of tobacco smoke have been demonstrated on the bactericidal activity of the alveolar macrophage of human and animal origin (M. Cutting, R. Laguarda, W. Pereira, J. Mullane, and G. Huber, Clin. Res. 23:705A, 1975; G. A. Warr, L. O. Gentry, and R. R. Martin, Clin. Res., 23:53A, 1975; 14).

Our data in Table 6 indicate that a reduction in leukocyte chemotaxis occurred after smoking unfiltered cigarettes, but that a significant reduction was not present in smokers after overnight abstinence from cigarettes. It should also be noted in Table 6 that the variance among the test and control observations was significantly higher ($P < 0.01$, F test) in the smoking group than the nonsmoking group, which might indicate individual differences in response to tobacco smoking. Additionally, the spontaneous motility of the leukocytes in the smoking smokers was reduced but not significantly so in comparison to the abstaining or nonsmokers. A similar transient defect in chemotaxis has not been found in the pulmonary macrophages of cigarette smokers, even though it is likely that these cells are exposed to higher concentrations of cigarette smoke components than are the peripheral blood leukocytes. On the contrary, the chemotactic responsiveness of the pulmonary macrophage is reported to be much greater in smokers than in nonsmokers (26). Thus, the chemotactic re-

sponse of the two cell populations may differ in their susceptibility to some cigarette products. Alternatively, metabolic conversion of the cigarette products may produce a circulating toxin which is inaccessible to the intraluminal pulmonary macrophage. Another unexplored possibility would be the presence of chemotactic factor inhibitor in the serum of the smokers (2). Finally, increased concentrations of cyclic 3',5'-adenosine monophosphate (AMP) adversely affect chemotaxis of rabbit polymorphonuclear leukocytes (I. Rivkin and E. L. Becker, Fed. Proc. 31:657, 1972; 18). This hormone may be active in cigarette smokers since nicotine, a potent stimulator of catecholamine release, transiently increases cyclic AMP in the isolated cat adrenal gland and its perfusate (17). Adverse effects of cyclic AMP have been demonstrated on the phagocytic capacity of leukocytes. Dibutyryl cyclic AMP in concentrations of 3 mM has been shown to decrease slightly phagocytosis of *Candida albicans* by human leukocytes (3) and starch particles by guinea pig polymorphonuclear leukocytes (10). No such adverse effect was present in our study. Thus, the effect of tobacco smoking on human leukocyte cyclic AMP is uncertain and suggests a direction for further research.

TABLE 6. *Leukocyte chemotaxis in smoking and nonsmoking subjects[a]*

Group[b]	Test	Control	Difference in distance moved
SS (12)	85.0 ± 26.3	75.4 ± 22.7	9.6 ± 10.3
AS (12)	103.7 ± 35.9	83.7 ± 26.9	20.0 ± 11.5
NSm (8)	113.6 ± 5.9	88.2 ± 5.4	25.4 ± 6.0
SS vs. AS		NS	$P < 0.03$
SS vs. NSm		NS	$P < 0.01$
AS vs. NSm		NS	NS

[a] Mean ± standard deviation. NS, Not significant at the 0.05 level.
[b] Abbreviations as in Table 1.

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