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Effect of Tobacco Smoking on the Functions of Polymorphonuclear Leukocytes

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Eight tests investigating the function of circulating polymorphonuclear leukocytes were performed in 68 subjects, half of whom smoked at least 20 cigarettes per day. Comparison of the two groups allowed determination of the in vivo effect of tobacco smoke on the nonspecific defense system of the body. Ingestion ability, oxygen consumption, and bactericidal activity were normal in smokers. Myeloperoxidase and neutrophil alkaline phosphatase activities also were unchanged. The nitroblue tetrazolium reduction and the serum lysozyme levels were slightly increased in smokers. The capillary tube random migration, though, was depressed, and intensive smoking further aggravated this change. It is suggested that tobacco smoke acts directly on one (or several) unidentified target site of polymorphonuclear leukocytes. This impairment, demonstrated in vivo, probably plays a role in the genesis of the bronchopulmonary diseases so frequent in heavy smokers.

Many works have been previously devoted to studying the biological effects of tobacco. Some of them indicate that tobacco may have an influence on the defense mechanisms of the body. The immunological system may be altered at different levels: inhibition of lymphocyte transformation by mitogens (9); decreased primary and secondary humoral responses in mice exposed for long periods to cigarette smoke (21); and modification of the morphology, the metabolic activity, and the clearing property of alveolar macrophages (6). The number of circulating polymorphonuclear leukocytes (PMNs) is increased in smokers (7, 10). The functional study of PMNs has only been undertaken recently (4, 19). The present work shows some disturbances of PMN functions in smokers. Such anomalies may contribute to the susceptibility to infection of heavy smokers.

MATERIALS AND METHODS

Subjects tested. Venous blood was obtained from 68 individuals, all technicians and medical staff of the Rangueil Medical Center (Table 1). They were fully informed of the objectives of the study. The medical history was obtained, and a physical examination was done before taking the blood samples. Subjects with diseases able to alter the functions of PMNs, such as diabetes mellitus, rheumatoid arthritis, or tuberculosis, were eliminated from the study, as well as currently or recently infected subjects and individuals under treatment, with the exception of women taking oral contraceptives. The subjects were divided into two groups: those who did not smoke and were not particularly exposed to passive inhalation of cigarette smoke, and those who smoked at least 20 cigarettes per day. In the latter group, 28 subjects smoked filter cigarettes, among which 4 used Virginia tobacco and 24 used black tobacco. The six nonfilter cigarette users smoked black tobacco. Among the whole smoker population, 30 inhaled the tobacco smoke and 18 had smoked one cigarette within 1 h before blood withdrawal. All the blood samples were obtained between 8:15 and 8:45 a.m. from fasting subjects. The whole study was carried out between October 1976 and February 1977. To determine the effects of heavy smoking, the tests were performed in 10 smokers before and after smoking five consecutive cigarettes in a period not exceeding 60 min. The volunteers were not fasting to prevent the risks of digestive malaise.

Methods. RM. Random migration (RM) was tested on whole blood according to the capillary tube method of Ketchel and Favour (16). Two milliliters of venous blood was collected with 20 μl of calcium heparinate (Calciparine, Laboratory Roche, Neuilly sur Seine, France). Ten microhematocrit tubes (Clay Adams, Parsippany, N.J.) were filled to two-thirds capacity and centrifuged at 4,000 rpm (MSE, Crawley, England) for 4 min. The tubes were then placed vertically at 37°C for 4 h. The distance between the front of leukocytes and the erythrocytes level was measured microscopically on each tube. The result was expressed as the arithmetic mean of the distances measured in the 10 tubes.

Particle ingestion activity. The particle ingestion activity was expressed by the phagocytic index, ac-

TABLE 1. *Characteristics of the two populations of smokers studied for functional PMN activity*

Population	No. of subjects tested		Age (yr)	
	Males	Females[a]	Mean[b]	Range
Nonsmokers	2	32	30 ± 6	22–51
Smokers	14	20	31 ± 8	22–56

[a] The greater number of females was due to the recruitment conditions of subjects for this study. This unequal sex distribution was not significant since no difference between males and females was observed in functional properties of PMNs (unpublished data).

[b] Arithmetic mean ± one standard deviation.

cording to Brandt's technique (3). This index corresponded to the average number of yeasts (*Saccharomyces cerevisiae*) ingested per PMN. Eight milliliters of blood was collected in a plastic syringe with 0.2 ml of heparin at 1,000 U/ml (Laboratory Vitrum, Vitry sur Seine, France). Two milliliters of Plasmagel (Laboratory Roger Bellon, Neuilly, France) was added, and the syringe was placed vertically in an incubator at 37°C. Twenty minutes later, the supernatant was collected, and the number of PMNs was calculated from the number of leukocytes (Coulter Counter, model S) and the differential. A 0.4-ml amount of a suspension of heat-killed yeasts at a concentration of 40,000 per μl was added to a volume of leukocyte suspension to obtain a ratio of 8 yeasts to 1 PMN. After gentle shaking, the tube was incubated at 37°C for 20 min. Then, after adding 0.1 ml of 0.9% disodium-ethylenediaminetetraacetate the tube was centrifuged (200 × *g* for 5 min). The supernatant was discarded and the cells were resuspended. Two smears were stained with panoptic staining (May-Grunwald-Giemsa), and the results were read at a magnification of ×1,000. The number of PMNs which had ingested 0, 1, 2, 3, ... 7 yeast particles or more was established. The score obtained for 100 consecutive PMNs divided by 100 provided the phagocytic index.

NBT dye reduction. The nitroblue tetrazolium (NBT) dye reduction was studied according to the slightly modified quantitative technique of Baehner and Nathan (2). Twenty milliliters of venous blood was collected in a plastic syringe with 2 ml of 5% disodium-EDTA. Five milliliters of Plasmagel was added, and the syringe was placed vertically at 37°C for 20 min. The supernatant was collected in a plastic tube, which was centrifuged at 120 × *g* for 10 min after adding a double volume of 0.87% ammonium chloride. The cell pellet was washed twice in Krebs-Ringer buffer, pH 7.4 containing 200 mg/100 ml of glucose. The number of phagocytic cells (mature neutrophil granulocytes plus monocytes) was adjusted to 25,000 per μl. Three reaction tubes were prepared in parallel (tubes A, B, and C). Tubes A and C contained 0.4 ml of Krebs-Ringer buffer + 0.4 ml of 0.1% NBT solution (BDH Chemicals Ltd., Poole, England) in 0.9% sodium chloride + 0.1 ml of 0.01 M potassium cyanide. Tube B contained 0.39 ml of Krebs-Ringer buffer + 0.01 ml of latex particles of 0.794-μm diameter (Serva, Heidelberg, West Germany) + 0.4 ml of NBT + 0.1 ml of potassium cyanide. A 0.1-ml sample of leukocyte suspension was added to each reaction tube after 15 min of preincubation in a waterbath at 37°C. The reaction was stopped by adding 10 ml of 0.5 N HCl to each tube: after 10 s of incubation for tube C and after 15 min for tubes A and B. The supernatant was discarded after 15 min of centrifugation at 1,000 × *g* at 4°C. The reduced NBT was extracted after adding 4 ml of pyridine (RP Prolabo, Paris, France) and boiling for 15 min. After centrifugation the optical density (OD) of tubes A and B was registered with a Beckman model 24 spectrophometer at 515 nm with the tube C extract as a blank. The tube A OD value represented the NBT reduction of resting phagocytes (resting OD), that of tube B represented the NBT reduction in stimulated phagocytes (latex OD), and the difference between these two values (ΔOD) represented the potential of metabolic stimulation of the phagocytes.

Oxygen consumption. The oxygen consumption of leukocytes during phagocytosis was registered with a Clark-type electrode (Gilson oxygraph, Gilson Medical Electronics, Middleton, Wis.) in a 2-ml thermostated water-jacketed cell with permanent magnetic stirring. The leukocyte suspension preparation was the same as for the NBT reduction test (see above), and the number of phagocytes was adjusted to 40,000 per μl. The cell was filled with Krebs-Ringer buffer and heated to 37°C. A total of 500 μl was then replaced by an equal volume of leukocyte suspension, reaching a final concentration of 10,000 phagocytes per μl. After stabilization at the 100% level, 20 μl of latex particles (Serva) was added. The oxygen consumption was graphically recorded at a speed of 3 cm/min with full-scale sensitivity. Two values were calculated on each graph: the percentage of oxygen consumed during the first 3 min after latex addition and the time necessary to consume 50% of the initial oxygen quantity.

Whole-blood bactericidal activity. The whole-blood bactericidal activity was measured by using the method of Castro et al. (5). Sterile technique was used throughout. Ten milliliters of venous blood was collected in a sterile flask containing 1 mg of heparin (Laboratory Roche). A 4.5-ml sample was then transferred to a 25-ml Erlenmeyer flask and kept in a water bath at 37°C until ready for use. Serum was obtained by centrifugation of the remaining 5.5 ml for 15 min at 1,200 × *g*. Two milliliters of this serum was placed in another 25-ml Erlenmeyer flask. The test bacteria were *Staphylococcus aureus* grown on the surface of a blood agar plate; stock cultures were subcultured every 2 weeks. For each experiment bacteria were grown overnight in Trypticase soy broth (BBL) and serially diluted in normal saline to obtain approximately 5 × 10^4 colony-forming units/ml in the final suspension. Each dilution was thoroughly shaken for 60 s. The test system was prepared by adding a sample of the *Staphylococcus* suspension to each Erlenmeyer flask: 0.5 ml for the whole blood and 0.2 ml for the plasma. Immediately after inoculation, the two flasks were shaken in a Vortex mixer to obtain maximum dispersion of the bacteria. Samples of 0.1 ml from each system were then incorporated into agar pour plates to determine the zero-time bacterial counts. An additional 0.1-ml sample from the whole-blood test system was lysed by placing it into 1 ml of distilled water in

a petri dish before agarose incorporation. The inoculated whole blood and serum were incubated in a water bath at 37°C for 1 h. At 10, 30, and 60 min, the same procedure was repeated for the whole blood, and it was repeated for the plasma after only 1 h. All agar plates were incubated for 24 h at 37°C. The colonies were counted, and the results were expressed as the percentage of *S. aureus* found at zero time.

Serum lysozyme. The serum lysozyme was assayed by the microbiological method, using *Micrococcus lysodeikticus* as substrate and hen egg white lysozyme as reference (Seratest Lysozyme, Laboratory Eurobio, Paris, France). Six dilutions ranging from 50 to 5% were prepared from a lysozyme stock solution (40 μg/ml). The effect of each dilution on an *M. lysodeikticus* suspension was registered on a spectrophotometer (Beckman model 24) at 580 nm. From these six curves a reference line was drawn permitting conversion of the OD values into lysozyme concentrations. For the determination itself, 0.3 ml of the serum to be tested was added to 0.3 ml of the *Micrococcus* suspension and gently mixed. After 30 s, the OD decrease was registered for 2 min. The ΔOD (difference between the first and last values of the 2-min period) was compared to the reference line, and the serum lysozyme value was expressed in micrograms of hen white egg lysozyme per milliliter of the serum.

Leukocytic cytochemical reactions. Two leukocytic cytochemical reactions were performed on blood smears. The neutrophil alkaline phosphatase was revealed by the α-naphthyl phosphate technique followed by the diazo reaction (Fast Garnet GBC salt) modified from Gomori's method. The results were expressed as a score, using the Kaplow technique for microscopic evaluation (18). The myeloperoxidase was shown with the Graham-Knoll technique (11) by the oxidation of benzidine from hydrogen peroxide. A score was established by giving each PMN a value ranging from 0 to 3, so that the score of each reaction may theoretically range from 0 to 300.

Cell counts and leukocyte differentials. Cell counts and leukocyte differentials were performed for each subject at the time of the PMN functional tests: cell counting with a Coulter Counter (model S) and differential by microscopic examination of peripheral blood smears stained with May-Grunwald-Giemsa stain.

Statistical data. Comparison of the means was interpreted according to the Student's *t* test. The differences were considered to be significant when the probability (*P*) was ≤0.05.

RESULTS

The results of the functional tests are presented in Tables 2 and 3. Phagocytic ability, oxygen consumption, and bactericidal activity for *S. aureus* were normal in subjects who smoke more than 20 cigarettes per day. The myeloperoxidase and neutrophil alkaline phosphatase activities were unchanged also.

As for the NBT reduction, the values obtained for resting (resting OD) and stimulated (latex OD) phagocytes were not significantly different from those of nonsmokers. However a borderline increase of the ΔOD was noted by comparison with the controls ($t = 1.95$; $P \simeq 0.05$).

TABLE 2. *Results of the RM, NBT reduction, and serum lysozyme in smokers and nonsmokers*[a]

Population	RM	NBT reduction[b]	Serum lysozyme
Nonsmokers (34)[c]	1.83 ± 0.16	0.358 ± 0.078	9.56 ± 3.43
Smokers (34)[c]	1.67 ± 0.43	0.395 ± 0.081	11.23 ± 3.40
P	0.05 < *P* < 0.02	≃0.05	<0.05

[a] Statistical significance (*P*) is presented for each pair of values.

[b] These results express the ΔOD (see Materials and Methods).

[c] The number of subjects tested is given in parentheses.

The capillary tube RM test was moderately but significantly decreased, and the serum lysozyme level was slightly higher than in nonsmokers.

The phagocytic index and the NBT reduction were not influenced by consecutive smoking of five cigarettes. The RM test, though, was markedly impaired, more than under usual smoking conditions (Table 4). No significant variation in PMN number was observed during this intensive smoking test.

Carboxyhemoglobinemia was detected in only three subjects. They all had smoked a cigarette within the hour preceding blood sampling. Carbon monoxide levels varied from 0.90 to 1.80 ml/100 ml of blood. The means of the results obtained in these three subjects did not show any difference from those of the whole population of smokers.

Four subjects stated that they did not inhale the cigarette smoke. The results of the RM test were normal in these subjects (1.90 ± 0.22), but the other results were identical to those of the other smokers (data not shown).

No difference was found for the mean value of each test between the 19 subjects who had smoked at least one cigarette before blood was taken and the 15 subjects who had not smoked since the night before (data not shown).

Only six subjects smoked cigarettes without filters (black tobacco in all cases). The results obtained for the latter were the same as for the whole population of smokers (data not shown).

DISCUSSION

The techniques used in this work test different phases of the functionnal activity of human circulating blood PMNs: migration without any chemotactic stimulus (RM), ingestion of solid materials (phagocytic index), triggering of certain redox systems (NBT reduction), and oxygen

TABLE 3. *Results of phagocyte index (PI), oxygen consumption, bactericidal activity, cytochemical myeloperoxidase (MPO) and neutrophil alkaline phosphatase (NAP) activities, and absolute numbers of circulating PMNs and phagocytes in smokers and nonsmokers*

Population	PI	O_2 consumption[a] (%)	Bactericidal activity[b] (%)	MPO[c]	NAP[c]	PMNs[d] (no./μl)	Phagocytes[d] (no./μl)
Nonsmokers (34)[e]	4.39 ± 1.07	35.9 ± 9.9	22.5 ± 18.8	259 ± 44	119 ± 57	3,940 ± 1,750	4,340 ± 1,860
Smokers (34)[e]	4.30 ± 1.05	35.6 ± 12.9	20.4 ± 13.8	255 ± 37	109 ± 41	4,530 ± 1,325	5,060 ± 1,360

[a] Percentage of oxygen consumption measured with the Clark electrode in 3 min.
[b] Percentage of viable *S. aureus* growing from the hemolyzed blood bacteria system after 120 min of incubation.
[c] Scores of MPO and NAP established as described in Materials and Methods.
[d] Absolute number per microliter of venous blood.
[e] The number of subjects tested is given in parentheses.

TABLE 4. *Results of the RM, phagocytic index (PI), and NBT reduction tests before and after smoking five consecutive cigarettes (10 smokers)*

Determination	RM[a]	PI	NBT reduction		
			Resting OD	Latex OD	ΔOD
Before	1.72 ± 0.26	4.09 ± 1.15	0.034 ± 0.010	0.406 ± 0.041	0.372 ± 0.039
After	1.04 ± 0.40	4.02 ± 1.25	0.026 ± 0.012	0.396 ± 0.035	0.370 ± 0.034

[a] $P < 0.001$.

consumption during phagocytosis. In the absence of a plasmatic bactericidal activity due to antibiotics, and an excessive number of leukocytes, the whole-blood bactericidal activity, as studied with the technique of Castro et al., reflects well the bactericidal capability of neutrophil PMNs (5). Myeloperoxidase plays an important role in the constitution of the oxygen-dependent bactericidal complex (17), and the neutrophil alkaline phosphatase level is increased in bacterial diseases (8). The serum lysozyme determination, with the exception of some blood disorders and renal failure, constitutes an excellent index of the bone marrow granulocyte turnover (13). The decrease of the RM found in the present work may be paralleled with the results obtained by Noble and Penny (19), on the one hand, and Bridges et al. (4), on the other hand. The former, using a modification of the Boyden method, observed a depression of leukocyte chemotaxis in 14 smoking subjects when compared to 13 nonsmoker controls. Moreover, results returned to normal when tests were performed after overnight abstention from smoking. The results of this work are very similar to ours. Bridges et al. (4) have more recently shown that chemotaxis of human PMNs submitted in vitro to tobacco smoke (whole smoke, gas phase or hydrosoluble fraction of the gas phase only) was impaired. The test factors inhibited the RM too, as determined in the modified Boyden chamber used by these authors. Our results, after in vivo smoking of five consecutive cigarettes, clearly showed that migration inhibition was directly related to the effect of the tobacco smoke. In Bridges' work the PMN glucose metabolism was increased through the anaerobic pathway as well as the hexose monophosphate shunt (4). Such an activation may be related to the slight increase we observed in the NBT reduction test. This increase seems to constitute a basic state of the smokers' PMNs, not influenced by heavy smoking, as shown by consecutive smoking of five cigarettes. This metabolic acceleration may be related to the moderate increase of the number of circulating PMNs which was previously observed by Noble and Penny (19) and to the significant increase of the serum lysozyme level. These results might be due to an acceleration of granulocyte turnover, releasing in the peripheral blood younger and metabolically more active PMNs. The inducing factor for the accelerated turnover might be either a latent infection localized in the respiratory tract or tobacco smoke itself. However, the normal neutrophil alkaline phosphatase level in smokers would be rather against the hypothesis of an infectious mechanism.

Anderson et al. (1), using a different NBT reduction test, also observed an increase in subjects who smoked at least 11 cigarettes per day. In our work, the particle ingestion was not impaired regardless of the technique used (yeast or *S. aureus*) or the circumstances (basic or intensive smoking). We could not find in the literature any information about the effects of tobacco smoke on phagocytosis of circulating PMNs. One may note, however, that Harris et al. (14) found no impairment of phagocytosis of alveolar macrophages in smokers.

The whole-blood bactericidal activity and the myeloperoxidase levels of smokers' PMNs were normal. Identical results were found in PMNs

by Noble and Penny (19) who used the Castro technique as we have done in the present work, and in alveolar macrophages of either rats exposed to cigarette smoke for long periods (15) or active human smokers (14). However, conflicting results have been published about the bactericidal activity of alveolar macrophages since Green and Carolin (12) and Rylander (20) observed a decrease of this function.

In conclusion, our results clearly indicate that neutrophil functions are impaired in smokers: chiefly, the migration properties and, more slightly, the metabolic state. The mechanisms of the changes have to be clarified, but tobacco smoke might act directly on the cells. One could postulate that such an impairment would represent an additional factor of susceptibility to infection related to the respiratory tract of chronic smokers.

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