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In Vitro Effect of Tobacco Smoke Components on the Functions of Normal Human Polymorphonuclear Leukocytes

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The function of polymorphonuclear leukocytes (PMNs) has previously been shown to be impaired in smokers in comparison with healthy nonsmokers. Potent inhibition of PMN chemotaxis has been achieved with whole tobacco smoke, the gas phase of smoke, and a water-soluble extract of whole smoke. In the present work several aspects of PMN function were studied after exposure to water-soluble fraction of the particle phase of tobacco smoke collected on glass fiber filters. These tests included capillary tube random migration, chemotaxis under agarose, phagocytosis of yeasts, Nitro Blue Tetrazolium dye reduction, and whole-blood bactericidal activity. The water extract of the particle fraction of smoke had a high content of nicotine when compared with the levels achieved in plasma of smokers and a much lower concentration of aldehydes when compared with the gas phase of smoke. It had no cytotoxic effect and did not affect phagocytosis, oxygen consumption, or bactericidal activity. Nitro Blue Tetrazolium reduction of both resting and stimulated PMNs was significantly decreased only with the most concentrated solution. The tested solutions exerted a dose-related depressive effect on capillary tube random migration, whereas the random migration measured in the agarose chemotaxis test was normal. Nevertheless, the chemotactic response to a caseine solution was significantly decreased. The same tests were performed in the presence of several concentrations of a nicotine solution and the only test to be affected was the capillary tube random migration, and, that only at a very high concentration. The results of this study contribute to the more precise delineation of the extent of the dysfunction of PMNs exposed to tobacco smoke components and indicate that deleterious products are released from the particle phase of the smoke, which deposits all along the respiratory tree.

The function of human polymorphonuclear leukocytes (PMNs) is known to be affected by cigarette smoking. In vivo studies have shown the motility and oxygen consumption of PMNs in the human oral cavity (11) and the chemotaxis of leukocytes in the peripheral blood (16) to be depressed in smokers compared with nonsmokers. In a recent publication, our group demonstrated that the Nitro Blue Tetrazolium (NBT) reduction by circulating PMNs was slightly increased, whereas the capillary tube random migration was depressed, in direct relation to the number of cigarettes smoked (10). In vitro studies have already been devoted to the effects of the water-soluble fraction of cigarette smoke, whole tobacco smoke, and the gas phase of smoke on PMN chemotaxis (5). Their inhibitory effect was not related to cytotoxicity or inhibition of glucose metabolism. Later studies suggested that the unsaturated aldehydes were major contributors to the inhibitory properties of cigarette smoke (6).

In the present study we have tested the effects on PMN function of (i) a water-soluble extract taken from the particle phase of tobacco smoke and (ii) a solution of nicotine (PMN function was evaluated using the mean of six in vitro tests).

MATERIALS AND METHODS

Isolation of leukocytes. Leukocytes were isolated from the peripheral blood of informed volunteers (hospital personnel, ranging in age from 22 to 35 years). Volunteers were fasting, healthy nonsmokers who were not on medication, with the exception of oral contraceptives. Venous blood was collected on heparin or disodium ethylenediaminetetraacetate. When necessary, leukocytes were obtained by Plasmagel sedimentation (Laboratory Roger Bellon, Neuilly, France), followed by ammonium chloride lysis of residual erythrocytes. Cell viability as determined by the trypan blue exclusion method was always greater than 97% of the total PMN population. Two tests were performed directly on whole blood and did not require leukocyte isolation.

Functional leukocyte tests. Six tests investigating different phases of the functional activity of PMNs were used in the present work. Five of them were fully described previously in a paper dealing with the in vivo effects of tobacco smoking on PMNs (10); they are only briefly reported in the following.

Random migration. Random migration was tested on heparinated whole blood according to the capillary tube method of Ketchel and Favour (14). Ten microhematocrit tubes (Clay Adams, Parsippany, N.J.) were filled and centrifuged at 1,500 × *g* (MSE, Crawley, England) for 4 min. After 4 h at 37°C, the distance of migration of the leukocyte front was microscopically measured on each tube. The results were expressed as the arithmetic mean of the distances measured in the 10 tubes.

Phagocytic index. The phagocytic index was established according to Brandt's technique (4). PMNs were collected after Plasmagel sedimentation (Laboratory Roger Bellon) of heparinized venous blood and added to a suspension of heat-killed yeasts in a ratio 8:1 yeasts/PMN. After 20 min of incubation, disodium ethylenediaminetetraacetate was added, and after centrifugation the supernatant was discarded. Smears were stained, and the number of PMNs which had ingested, respectively, 0, 1, 2, ... 7 or more yeast particles was microscopically established. The score obtained for 100 consecutive PMNs divided by 100 provided the phagocytic index.

NBT dye reduction. NBT dye reduction was studied according to the quantitative technique of Baehner and Nathan (2). After Plasmagel sedimentation of disodium ethylenediaminetetraacetate anti-coagulated venous blood and erythrocyte lysis in ammonium chloride, the cell pellet was washed twice in Krebs-Ringer (KR) buffer, and the number of phagocytes was adjusted to 25,000 per μl. Three reaction tubes were prepared in parallel (tubes A, B, and C). Tubes A and C contained NBT solution (BDH Chemicals Ltd., Poole, England), potassium cyanide, and KR buffer. Moreover, tube B contained latex particles (Serva, Heidelberg, West Germany). A sample of the leukocyte suspension was then added to each tube. The reaction was stopped by adding HCl after 10 s of incubation for tube C and after 15 min for tubes A and B. The supernatant was discarded, and the reduced NBT was extracted in boiling pyridine (RP Prolabo, Paris, France). The optical density of tubes A and B was registered at 515 nm with the tube C extract as a blank, representing, respectively, the levels of NBT reduction in resting phagocytes (resting optical density) and in stimulated phagocytes (stimulated optical density); the difference between these two values represented the potential of metabolic stimulation of the phagocytes.

Oxygen consumption. The oxygen consumption of the leukocytes during phagocytosis was registered with a Clark-type electrode (Gilson oxygraph, Gilson Medical Electronics, Middleton, Wis.). The leukocyte suspension was the same as for the NBT test (see above). After stabilization at the 100% level, latex particles (Serva) were added, and the percentage of oxygen consumed during the first 3 min was calculated on each graph.

Whole-blood bactericidal activity. The whole-blood bactericidal activity was measured by the method of Castro et al. (7). Venous blood was collected in a sterile heparinized flask. A sample was conserved, and the rest was centrifuged to obtain plasma. Samples of a *Staphylococcus aureus* suspension at a final dilution of approximately 5×10^4 colony-forming units/μl were added to Erlenmeyer flasks. Immediately after inoculation, samples from each flask were incorporated into poured agar to determine the zero-time bacterial counts. An additional sample from the whole-blood test system was lysed in distilled water before agar incorporation. The inoculated whole-blood and plasma samples were incubated for 2 h. At 30, 60, and 120 min, the same procedure was repeated. All agar plates were incubated for 24 h at 37°C, and the results were expressed as the percentage of *S. aureus* found at zero time.

Chemotaxis. The chemotactic activity of PMNs was studied under agarose, using the method of Nelson et al. (15). Agarose (Industrie Biologique Française, Genevilliers, France) was prepared as a 10% solution in Dulbecco-modified Eagle medium (Eurobio, Paris, France) at a pH of 7.2 and poured into petri dishes. In each petri dish, six groups of three linear wells were made in agar. Each well was 2.4 mm in diameter and 2.4 mm from the adjacent wells. Leukocytes were sedimented on Plasmagel and brought to a concentration of 55,000 per μl. Five microliters of this suspension was placed in each central well. The lateral wells were filled, respectively, with 5 μl of Eagle medium solution and 5 μl of a casein solution (Difco, Detroit, Mich.) at a concentration of 40 mg/ml in 20% fresh serum. The petri dishes were then incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO_2 in air. After methanol and formol fixation, the agarose layer was removed, and the dishes were stained with May-Grunwald-Giemsa stain and air dried. The migration distances measured from the margin of the well toward the chemotactic factor (distance A) and the control medium (distance B), respectively, were microscopically measured (×160 magnification). The results were expressed as a chemotactic index (A/B).

Tobacco smoke. Nonfilter cigarettes of black tobacco kindly supplied by the Service d'Exploitation Industrielle des Tabacs et des Allumettes (SEITA, Paris, France) were used for all experiments. Cigarettes were 7 cm long and weighed 1 g each. The cigarettes were smoked with a piston-type smoking machine for single cigarettes (Borgwaldt, Hamburg, West Germany), using a 30-ml puff volume of 2-s duration at 50-s intervals. The particle phase of the smoke was collected on glass fiber filters 44 mm in diameter. To prepare the water-soluble fraction of the particle phase of the smoke (WSF), five cigarettes were consecutively smoked on one filter. The filter was then incubated for 16 h in 10 ml of KR buffer, and the solution was filtered on a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.) to eliminate the particles which remained in suspension. Fresh stock WSF solution was prepared each day of experiment.

To establish the characteristics of the solution, the pH and the optical density were measured in each newly prepared solution. A linear relation between the concentration of the WSF solution and the optical density at a wavelength of 502.4 nm (Beckman spec-

trophotometer, model 24) was used as a control of the reproducibility of the procedure. The nicotine concentration was determined according to the standard Coresta method (8) in the different WSF preparations. The aldehyde content (i.e., acetaldehyde, propionaldehyde, crotonaldehyde, and furfural) was measured by gas chromatography (Hewlett-Packard 5710A), using three of the WSF solutions. Aqueous solutions of these aldehydes (Aldrich Chemical Co., Milwaukee, Wis.) were used as reference standards.

Three concentrations of the WSF solution were used (a stock solution and 10^{-1} and 10^{-2} dilutions in KR buffer). The appropriate dilutions were added to whole blood and incubated for 30 min at 37°C before the start of the assay or the isolation of leukocytes. Furthermore, for the chemotaxis test, the WSF solution was directly added to leukocytes collected from blood not previously incubated with the tobacco smoke solution. In all of the tests, the volume of solution added was calculated to provide a dilution of 1:6.

To take into account the effects of dilution and acidification of the whole blood by WSF solution, reference tests were performed after incubation with an equal volume of KR solution and acidified KR solution, pH 7.07.

Nicotine solution. Because it could not be solubilized in KR buffer, nicotine (Aldrich Chemical Co.) was diluted in 0.9% sodium chloride to obtain final concentrations in whole blood of 1.85, 18.5, and 185 μmol/liter. These dilutions were used as described above for the WSF solution.

Viability and morphology of PMNs. After incubation in the WSF solution, viability was controlled by trypan blue exclusion, and morphology of PMNs was studied by electron microscopy according to the standard method currently used for blood cell examination.

Statistical analysis of data. Comparison of the means was interpreted according to Student's t test applied to short series. The differences were considered to be significant when the probability was <0.05.

RESULTS

Characteristics of the WSF of the particle phase of tobacco smoke. The mean values for the pH, optical density, and nicotine and aldehyde content of the WSF solutions used in the tests are shown in Table 1. No effect on cell morphology could be observed by electron microscope studies, and the PMN viability was greater than 93% as measured by trypan blue exclusion.

Effects of the WSF of the particle phase of tobacco smoke on PMN functions. The effects of the WSF solution on PMNs are given in Table 2. The phagocytic activity of PMNs versus killed *Saccharomyces cerevisiae* and living *S. aureus* (tested during the bactericidal assay) was normal. The oxygen consumption by the stimulated cells showed no significant difference when compared with PMNs previously incubated in KR buffer. The whole-blood bactericidal activity was normal, and the plasma exhibited no bactericidal activity.

TABLE 1. *Characteristics of the WSF of the particle phase of tobacco smoke*

Measured parameter	No. of tests	Mean ± standard error
pH	30	7.08 ± 0.01
Optical density (502.4 nm)	30	0.409 ± 0.020
Nicotine (mg/ml)	10	0.157 ± 0.003
Acetaldehyde (mmol)	3	0.67 ± 0.060
Propionaldehyde (mmol)	3	0.11 ± 0.026
Furfural (mmol)	3	0.222 ± 0.039

Conversely, three tests showed significant modifications. The capillary tube random migration was very significantly depressed, whereas the spontaneous migration tested in the agarose chemotaxis assay was normal (0.75 ± 0.12 versus 0.69 ± 0.10 for controls). The decrease of capillary tube random migration was dose related (see Fig. 1), the effect being significant even with the 10^{-2} dilution ($P < 0.001$). The chemotactic response to caseine was greatly decreased (Table 3). This decrease was greater when leukocytes were incubated with the WSF solution in the central well than with leukocytes collected from whole blood previously incubated with WSF solution ($P < 0.02$). Moreover, the chemotactic response of normal PMNs to a chemoattractant solution prepared with WSF was significantly decreased. The WSF solution alone without caseine and serum exerted a "repulsive effect" on normal PMNs. In the latter condition, the distance of spontaneous migration (toward the B well) was greater than in the other systems ($0.02 < P < 0.05$).

The WSF solution also exerted a depressive effect on the NBT dye reduction observed with both resting and stimulated PMNs (Table 2). Inasmuch as the tobacco smoke solution prepared with KR buffer was acidic (pH 7.08), the effect of acidity on the level of NBT reduction was tested: no difference was observed between NBT reduction ability of PMNs collected from standard KR- and acidified KR-preincubated whole blood (data not shown). In a series of 10 experiments it appeared that the 10^{-1}-diluted WSF solution had no effect on NBT reduction by PMNs (Table 4).

Effects of nicotine solution on PMN functions. The nicotine solutions at any concentration had no effect on the phagocytic index, the NBT reduction, the oxygen consumption, the bactericidal activity, or the chemotactic response (data not shown). The capillary tube random migration was only significantly depressed at the 185-μmol/liter concentration (1.57

TABLE 2. *Effects of the WSF of the particle phase of tobacco smoke on PMN functions*[a]

Test	No. of tests	Solution		Significance (*P*)
		KR buffer	WSF	
Random migration (mm/4 h)	24	1.59 ± 0.14	0.89 ± 0.45	<0.001
Chemotactic index	12	1.80 ± 0.14	1.45 ± 0.15	<0.001
Phagocytic index	10	5.04 ± 0.37	5.02 ± 0.75	NS[b]
NBT reduction	10			
Resting OD		0.031 ± 0.009	0.020 ± 0.009	<0.02
Stimulated OD		0.404 ± 0.031	0.324 ± 0.038	<0.001
ΔOD		0.373 ± 0.033	0.304 ± 0.034	<0.001
O_2 consumption (% for 3 min)	10	30.93 ± 13.14	33.40 ± 11.10	NS
Bactericidal activity (*S. aureus*; % live bacteria at 120 min)	10	20.1 ± 11.5	16.4 ± 9.8	NS

[a] Each series of tests was performed according to the standard technique presented in the text, after 30 min of incubation at 37°C of a volume of the given solution so as to obtain a one-sixth dilution in whole blood. Values are given as means ± standard errors. OD, Optical density.

[b] NS, Not significant at $P = 0.05$.

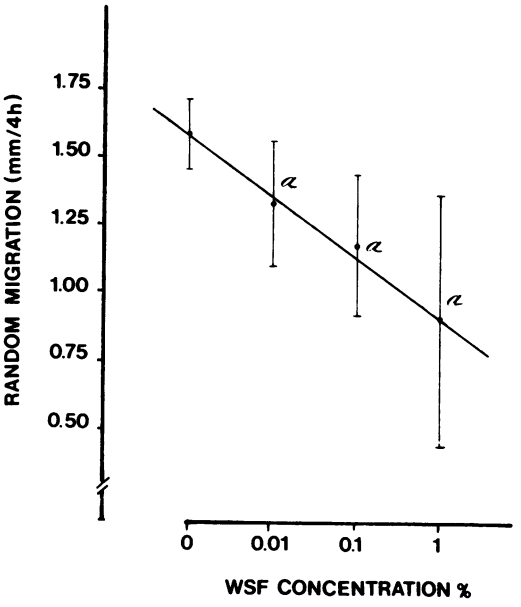

FIG. 1. *Effects of various concentrations of WSF solution on capillary tube random migration of PMNs. The zero concentration means that the whole blood was incubated for 30 min at 37°C with acidified KR buffer before the test. The results represent the mean percentage (± standard error) of 14 experiments. a = mean percentages significantly (P < 0.001) different from zero concentration values.*

± 0.37) when compared with the reference tests performed in the presence of 0.9% sodium chloride (1.94 ± 0.27). There was only a slight nonsignificant decrease at lower concentrations (data not shown). Since the pH of the most concentrated nicotine solution and that of the 0.9% sodium chloride solution were different (10.12 and 6.35, respectively) we verified the influence of pH on capillary tube migration. No difference was observed between two sets of samples preincubated in 0.9% sodium chloride solutions at pH 10.12 or 6.30.

DISCUSSION

In a previous paper (10), we described significant changes in PMN function in smokers as determined by NBT dye reduction and random migration studies. The present study was intended to test the in vitro effect of a water-soluble extract of the particle phase of tobacco smoke collected in glass fiber filters. The method of preparation of our extract was different from that of Bridges et al. (5), who used smoke collected in a flask containing distilled water at 2°C, i.e., the WSF of the gas phase of the whole smoke. Conversely, we tested the WSF of the particle phase of smoke. The particle phase of the smoke contains volatile substances and various particles which represent 5 to 10% of the weight of tobacco smoke. The size of particles ranges from 0.1 to 1 μm, which allows their deep penetration and deposition in both the upper and lower respiratory tract, where they exert a harmful effect on alveolar macrophages (9). The solution we prepared had a nicotine concentration about 1,000 times higher than what is found in the plasma of smokers, according to the results of Armitage et al. (1). Thus, the 10^{-2} dilution of the WSF was still above the "physiological" concentration of plasma nicotine. The concentrations of acetaldehyde, furfural, and propionaldehyde were, respectively, 8, 2, and 13 times lower than the concentrations which would be found in the smoke of five cigarettes, according to the results of Bridges et al. (6). Moreover, the final concentrations of these al-

TABLE 3. *Effects of the WSF solution on PMN chemotaxis (12 experiments)*

Medium for whole-blood preincubation	Medium for suspension of isolated leukocytes	Chemoattractant system	Distance of migration[a] (mm)		Chemotactic index (A/B)
			A	B	
KR	KR	KR-Cas-S[b]	1.25 ± 0.23	0.69 ± 0.10	1.80 ± 0.14
WSF	KR	KR-Cas-S	1.09 ± 0.26	0.75 ± 0.12	1.45 ± 0.15
KR	WSF	KR-Cas-S	1.01 ± 0.15	0.77 ± 0.07	1.30 ± 0.13
KR	KR	WSF-Cas-S	0.95 ± 0.23	0.70 ± 0.08	1.35 ± 0.20
KR	KR	WSF	0.81 ± 0.32	0.95 ± 0.36	0.84 ± 0.05

[a] Distance of migration of PMNs under agarose toward A and B wells; means ± standard errors.
[b] Cas, Caseine; S, serum.

TABLE 4. *Effects of various concentrations of WSF solution on NBT dye reduction of PMNs*

NBT reduction (10 expt)	Medium of whole-blood preincubation		
	KR	WSF	
		Pure	0.10 Dilution
Resting OD[a]	0.053 ± 0.017[b]	0.037 ± 0.015	0.047 ± 0.017
Stimulated OD	0.379 ± 0.036	0.333 ± 0.051	0.373 ± 0.070
ΔOD	0.326 ± 0.031	0.296 ± 0.049	0.326 ± 0.060

[a] OD, Optical density.
[b] Mean ± standard error.

dehydes in the reaction tubes were much smaller than those used by Bridges et al. (6) to study the effects of smoke components on PMN chemotaxis and glucose metabolism. As no deleterious effect was noted on the viability and ultrastructure of PMNs, the functional anomalies described herein may be considered as the expression of metabolic disturbances induced by tobacco smoke components present in the WSF.

The decrease of capillary tube random migration observed with the WSF is very similar to the results we obtained previously in a group of smokers (10). In that work, it was shown that migration inhibition was directly related to the dose of the tobacco smoke and, similarly, there was a dose-related effect of the WSF on random migration. This inhibition may be partly due to the nicotine alone since its concentration in whole blood (160 μmol/liter) was equivalent to that of a pure nicotine solution possessing the same inhibitory effect (185 μmol/liter). However, other factors are probably involved since the inhibition was also observed in smokers whose nicotine levels are substantially lower than those used in our experimental protocol. There exists a discordance between the values of random migration obtained with the agarose and capillary tube techniques. In fact, in our experience we never found any parallelism in the results of those two techniques. It may be partly explained by the fact that, in the capillary tube method, the PMNs remain under the effect of plasma factors during their vertical migration, whereas in the agarose technique the washed PMNs are deprived of the influence of plasma factors during their horizontal migration. Nevertheless, the normal results of agarose random migration of PMNs in contact with WSF, either during preincubation in whole blood or during the test period (Table 3), indicate that the tobacco smoke components exert no direct effect on PMNs. These data do not agree with those of Bridges et al., who found that their test agents at concentrations that inhibited directional migration were shown to be equally effective as inhibitors of random migration (5). Such a difference may depend on both the test agents and the techniques used.

As previously described by Bridges et al. (5) with a different experimental procedure, we observed a very significant decrease in chemotaxis in the two systems used (preincubation of whole blood and incubation of isolated leukocytes). This defect is not due to a cellular dysfunction affecting the spontaneous motility of PMNs since random migration was found to be normal in the same test (see above). It was verified that the WSF alone had no chemotactic activity, and moreover, during this test we obtained a negative chemotactic index. This means that the cells on the leading edge of the central well were settled, allowing a greater number of PMNs to

migrate on the other side, i.e., toward the control buffer-filled well, thus permitting a longer spontaneous migration distance. Such a pattern of chemotaxis was also obtained in our laboratory with high concentrations of casein (unpublished data). This phenomenon may be related to the "high-dose inhibition of chemotaxis" recently described by Fehr and Dahinden (13). For these authors the chemotactic hyporesponsiveness would be a consequence of increased cell adhesiveness with correlated stimulation of the hexose monophosphate pathway activity. Such an increase of glucose metabolism was observed by Bridges et al. when PMNs were exposed to their WSF of tobacco smoke (5). Although the WSF had no chemotactic activity, it might act according to the model of chemotactic deactivation recently proposed by Spilberg et al. by interacting with microtubule organization in the PMNs (19).

The decrease of NBT reduction with both resting and stimulated PMNs was observed only with the pure WSF. This effect was not nicotine dependent. This therefore means that a component of tobacco smoke when highly concentrated is able to interfere with the superoxide anion-generating systems of PMNs. The observed decrease in NBT reduction was not related to an abnormal rate of oxygen consumption, the latter being normal even in the presence of the highest concentrations of WSF; neither did it interfere with the whole-blood bactericidal activity, whereas Shea et al. have recently shown that experimental tobacco smoke inhalation in rats could depress the in vitro bactericidal function of alveolar macrophages (18). From a practical point of view, our tobacco smoke extract, when tested at physiological concentrations, exerted no effect on NBT reduction capability of PMNs. This is not in contradiction with the slight increase of NBT reduction that we previously observed in smokers and does in fact reinforce the concept that there are factors acting indirectly on PMNs, which may be accelerating granulocyte turnover (10).

Complementing our previous in vivo studies, the results of this in vitro study strengthen the concept that tobacco smoke manifests a harmful effect on PMN function. PMN motility is the most severely altered function, whereas other functions such as phagocytosis and bactericidal activity are not impaired. Metabolic activities are affected only with concentration levels of tobacco smoke components (WSF) which are not attained in the blood of smokers. Nevertheless, much higher concentrations of tobacco smoke components, both gaseous and particulate, may be present in the oral cavity and the respiratory air spaces. At such concentrations more deleterious effects may be observed, including the release of lysosomal hydrolases from local PMNs (3, 12). Thus, PMNs would be doubly involved in tobacco-related pathological manifestations: (i) by participating in conjunction with alveolar macrophages to damage the lung tissue (3, 17), and (ii) by their migratory impairment which contributes to the weakening of both the local and systemic host defense mechanisms. Further studies are presently being performed in our laboratory to define the cellular mechanisms involved.

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