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Effects of Tobacco Smoke on Chemotaxis and Glucose Metabolism of Polymorphonuclear Leukocytes

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The effect of tobacco smoke on in vitro chemotaxis of human polymorphonuclear leukocytes (PMN) was determined. Whole tobacco smoke, gas phase of smoke, and water-soluble fraction were potent inhibitors of PMN chemotaxis. The results indicated that PMN chemotaxis was inhibited in a dose-dependent manner by water-soluble fraction and that this suppression was not a result of cytotoxicity. In an attempt to determine the mechanism of chemotaxis inhibition, the effect of tobacco smoke on glucose metabolism of PMN was studied. Exposure of PMN to whole smoke, gas phase, or water-soluble fraction resulted in an increase (twofold) in glucose catabolism via both glycolysis and the hexose monophosphate shunt, with no apparent effects on the metabolism of glucose via the tricarboxylic acid cycle. These results suggest that the inhibitory effects of tobacco smoke on PMN chemotaxis were not directly attributable to effects on glucose metabolism of these cells. Further, the inhibitory effects of water-soluble fraction on PMN chemotaxis were shown to be largely irreversible and preventable in the presence of cysteine. Thus, the major inhibitory effects of tobacco smoke probably result from the direct action of oxidants and/or thiol-reactive substances on PMN.

The polymorphonuclear leukocyte (PMN) is the first line of host defense against acute bacterial infections. The initial event necessary for PMN to exert their scavenger function is directional migration to the target (chemotaxis). In a number of clinical conditions, defective chemotactic responsiveness of PMN has been accompanied by an increased susceptibility of the host to recurrent bacterial infection (6, 23, 29).

Tobacco smoke has been shown to severely affect PMN function. Exposure of oral PMN to whole tobacco smoke in situ caused an inhibition of both locomotion and phagocytosis (13). Tobacco smoke may also affect the cellular defense mechanisms at the systemic level. PMN obtained from adult, male cigarette smokers have been shown to exhibit a depressed chemotactic response as compared with PMN obtained either from the same subjects abstaining from cigarettes or from nonsmokers (25). It has also been observed that the directional migration of PMN isolated from human peripheral blood is inhibited in vitro by small concentrations of water-soluble (WSF) fraction of tobacco smoke (21).

The purpose of this study was to determine the effects of tobacco smoke and WSF of tobacco smoke on the in vitro chemotactic response of PMN obtained from human peripheral blood. Further, since the directional migration of PMN is energy dependent (9, 16, 19), the effect of tobacco smoke or its fractions on the glucose metabolism of PMN was investigated. Finally, the ability of cysteine to protect against tobacco smoke inhibition of PMN chemotaxis was tested. These studies were suggested by the previous demonstration that cysteine protected the alveolar macrophage from tobacco smoke inhibition of phagocytosis (17).

MATERIALS AND METHODS

Isolation of PMN. PMN were isolated from the peripheral blood of nonsmoking males ranging in age from 20 to 35 years. Venous blood (100 ml) was drawn at 8 a.m. in order to circumvent any changes in cellular metabolism due to diurnal rhythm (3). The healthy, nonmedicated volunteers were fasted for at least 12 h before venipuncture. PMN were obtained by dextran (T250, Pharmacia Fine Chemicals, Piscataway, N.J.) sedimentation of heparinized whole blood (10), separation of granulocytes from mononuclear cells by the Hypaque (Winthrop Laboratories, New York)-Ficoll (Sigma Chemical Co., St. Louis, Mo.) technique (8), and hypotonic saline lysis of residual erythrocytes (10). Cells were resuspended in Gey balanced salt solution containing 2% bovine serum albumin and sodium bicarbonate (Flow Laboratories, Rockville, Md.), and cell counts were performed with a Petroff-Hauser chamber. The concentration of PMN was adjusted to 3 ×

10^6 cells/ml by the addition of Gey medium. This isolation procedure resulted in PMN populations of greater than 98% cell viability as determined by trypan blue exclusion (28).

Tobacco smoke and tobacco smoke fractions. Whole tobacco smoke and gas phase were produced from the 1R1 reference cigarettes of the University of Kentucky Tobacco and Health Research Institute. Tobacco smoke was delivered by using a single-port, reverse smoking machine calibrated in half puffs (17.5 ml). The gas phase of tobacco smoke was produced similarly but using a Cambridge filter apparatus. Exposure of PMN to tobacco smoke was accomplished by blowing the smoke through a 5-ml cell suspension in Gey medium (3×10^6 PMN/ml). Control PMN suspensions were exposed to an equivalent volume of air delivered in a similar manner.

Fresh WSF of tobacco smoke was obtained from John Benner of the University of Kentucky Tobacco and Health Research Institute. WSF was prepared by smoking 85-mm-long 1R1 cigarettes on a Borgwaldt smoking machine, using a 35-ml puff volume of 2-s duration at 1-min intervals. Smoke was collected in a flask containing distilled water at 2°C. The concentration of WSF was determined as residue remaining after evaporation of a 5-ml portion at a pressure of 30 mm at 35°C with a 50-ml/min stream of nitrogen. The stock WSF usually had a residue concentration of 50 mg/ml. Appropriate dilutions were made in Gey medium before addition to cell suspensions.

Chemotaxis assay. Chemotaxis of PMN was tested using a modified Boyden chamber (7). The chemotactic substance was fresh human autologous serum activated with lipopolysaccharide B, *Escherichia coli* 0127:B8, endotoxin (Difco). The activated serum was prepared by mixing 2 ml of fresh serum with 1 ml of Gey medium containing 5 μg of endotoxin, incubating at 37°C for 60 min, and diluting the activated complement by the addition of 3 ml of cold (0°C) Gey medium.

Two membrane filters (mesh size 0.45 and 3 μm; Millipore Corp., Bedford, Mass.) were used to separate the two compartments of the Boyden chamber in order to avoid errors due to diminished adherence of PMN (20). The 0.45-μm filter was used on the attractant side of the chamber.

The cell suspension (3×10^6 PMN/ml) with or without addition of tobacco smoke products was placed in the upper compartment of the Boyden chamber, with the chemotactic agent in the lower chamber. The chambers were incubated for 2 h at 37°C in an atmosphere of humidified air containing 5% carbon dioxide. The filters were removed, stained with Ehrlich hematoxylin (7), cleared, and mounted with the attractant side up. The cells were counted in 10 consecutive, evenly spaced fields (defined by an ocular grid) on the attractant side of the 3-μm filter at ×400 magnification, using a Leitz SM-LUX microscope. Random movement as a cause for the chemotaxis results was ruled out in each experiment by suitable controls using Gey medium on the attractant side. In each experiment, chemotaxis assays were done in duplicate or triplicate. Chemotaxis results were highly reproducible within a given experiment, although there was considerable variability between leukocyte preparations from different subjects. To compare data among experiments, chemotaxis results were expressed as a ratio of experimental values to control values. These ratios were used in the statistical treatment of the data as indicated below. In some instances, the chemotaxis results are expressed in terms of percentage of control (ratio of experimental to control × 100).

Experiments testing the protection of cysteine against tobacco smoke inhibition of chemotaxis were done by the addition of tobacco smoke products to PMN suspensions containing 10 mM cysteine. The reversibility of the effects of tobacco smoke was also tested by: incubating PMN suspensions at 37°C for 15 min in the presence of WSF (1,000 μg/ml); separating the treated PMN from excess agents in solution by centrifugation; resuspending the PMN in Gey medium with and without 10 mM cysteine; and testing for chemotaxis as indicated previously.

Glucose metabolic studies. Incubation vessels were 10-ml Erlenmeyer flasks containing a center well. Each flask contained in the main compartment 3 ml of a cell suspension (3×10^6 PMN/ml in Gey medium containing 1 g of glucose/liter). To cell suspensions treated with whole tobacco smoke or gas phase was added 0.3 ml of Gey medium. The WSF was added to the cell suspensions in 0.3 ml of Gey medium. Vaccine stoppers were inserted into the flasks, and each flask was gassed through syringe needles with air containing 5% CO_2 for 3 min. Reactions were initiated by injecting through the vaccine stoppers 5 μl of either [1-^{14}C]- or [6-^{14}C]glucose (0.5 μCi, 10 nmol; New England Nuclear, Boston, Mass.). Reaction mixtures were incubated at 37°C with shaking for 90 min and were terminated by injecting 0.2 ml of 2 N HCl into the main compartment (final pH below 2). To each center well was added 0.2 ml of *p*-(diisobutylcresoxyethoxyethyl)dimethylbenzylammonium hydroxide (Packard Instrument Co., Inc., Downers Grove, Ill.), and incubation at 37°C was continued for an additional 30 min to insure complete trapping of any evolved $^{14}CO_2$ in the center well. Center well contents were then removed quantitatively and placed in counting vials, which contained 10 ml of a solution of 0.9% (wt/vol) 2,5-diphenyloxazole (PPO) and 0.1% (wt/vol) 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene (POPOP) (Packard Instrument Co., Inc.) in toluene. Vials were counted in a Packard Tri-Carb liquid scintillation spectrometer (model 3375). The contents of the main compartments were transferred to glass centrifuge tubes, and the supernatants were collected by decantation after centrifugation. The packed PMN were analyzed for glycogen. Protein was removed from the supernatants by the addition of 1 ml of 15% (wt/vol) tricholoroacetic acid and centrifuged. The acid supernatants were collected by decantation and analyzed for glucose and lactic acid. In each experiment, suitable controls lacking either PMN or test agents (e.g., tobacco smoke fractions, etc.) were included.

Glycogen, glucose, and lactic acid determinations. The glycogen content of PMN was determined by the method of Anderson and Stowring (1).

The glucose consumed by PMN was calculated by

difference between the nanomoles of glucose present in the incubation mixtures without PMN and the nanomoles of glucose remaining in test mixtures. Glucose was determined by using glucose oxidase (Glucostat, Worthington Biochemical Corp., Freehold, N.J.) and suitable aliquots (0.05 and 0.1 ml) of the acid supernatants, which were diluted to 2 ml with 0.5 M potassium phosphate buffer (pH 7.0).

Lactic acid was determined essentially according to the method of Barker and Summerson (4). Controls containing an equivalent concentration of test agent in the absence of PMN were used as blanks.

Glucose disappearance or lactic acid production were calculated in terms of nanomoles of glucose consumed or lactic acid produced per 90 min per 9×10^6 PMN, respectively. The expression of metabolic results in terms of PMN numbers was valid, since a cellular suspension containing 1×10^7 PMN/ml consistently produced a protein concentration of 1.18 ± 0.03 mg of protein per ml as determined by the method of Lowry et al. (22).

Although the results of the metabolic experiments were highly reproducible within a given experiment, there was some variability between leukocyte preparations from different individuals. Therefore, to compare data among experiments, the metabolic results were expressed as a ratio of the experimental values to the control values. Numerical values for this ratio of greater than one represented metabolic stimulation, whereas values of less than one represented metabolic inhibition.

Cytotoxicity of tobacco smoke. Tobacco smoke and its fractions at various concentrations were incubated with PMN suspensions (3×10^6 PMN/ml) in Gey medium for 2 h at 37°C. The viability of the PMN was measured by the exclusion of 0.1% (wt/vol) trypan blue as previously described (28). The supernatants obtained by centrifugation of the treated PMN suspensions were analyzed for lactic acid dehydrogenase (EC 1.1.1.27; LDH) and β-glucuronidase (EC 3.2.1.31) activities.

LDH activity was measured as described previously (5). The oxidation of reduced nicotinamide adenine dinucleotide (NADH) was followed continuously at 340 nm with a Unicam SP1700 ultraviolet spectrophotometer equipped with a Unicam AR 25 linear recorder. One unit of LDH activity is defined as the amount of enzyme required to catalyze the oxidation of 1 μmol of NADH per min.

Beta-glucuronidase activity was measured as previously described (14), using phenolphthalein glucuronide as substrate and an incubation period of 18 h. One unit of β-glucuronidase is defined as the amount of enzyme required to liberate 1 μmol of phenolphthalein.

Statistical analysis of data. All statistical analyses were *t* test, testing the hypothesis that the ratio of treated to control is equal to one. Significance levels are based on the log ratio. The log transformation was used to eliminate skewing in the distribution of the ratio.

RESULTS

Chemotaxis and glucose metabolism of untreated PMN. Presented in Table 1 are the results for chemotaxis, glucose utilization, lactic acid production, and hexose monophosphate (HMP) activity of untreated PMN. Although there was considerable variability from experiment to experiment as indicated by the range, values for these parameters were highly reproducible within a given experiment.

The glycogen content of PMN was negligible (data not shown), and thus the Gey medium was the major source of glucose for cellular metabolism.

The level of HMP activity, as measured by the release of $^{14}CO_2$ from [1-^{14}C]glucose, gave a mean value of 293 cpm, which accounted for less than 5.6 nmol of glucose being metabolized via this pathway per 9×10^6 PMN in 90 min of incubation.

The release of $^{14}CO_2$ from [6-^{14}C]glucose, a measure of the glucose catabolic rate via the tricarboxylic acid cycle, was negligible as compared with background (16.6 ± 6.0 cpm/9×10^6 PMN per 90 min). Further, WSF, whole smoke, and gas phase at concentrations inhibitory to chemotaxis had no demonstratable effect on $^{14}CO_2$ release from [6-^{14}C]glucose (data not shown). Thus, $^{14}CO_2$ release from [1-^{14}C]glucose was an accurate measure of the HMP activity, and was not a result of the complete oxidative metabolism of glucose via the tricarboxylic acid cycle.

The predominant route for the catabolism of glucose by PMN was the glycolytic pathway, which accounted for the utilization of approximately 900 nmol of glucose per 9×10^6 PMN in 90 min of incubation. Thus, the metabolism of glucose via glycolysis was nearly 180-fold greater than via the HMP pathway.

Effects of tobacco smoke on PMN chemotaxis. Whole tobacco smoke and the gas phase of tobacco smoke were shown to be potent inhibitors of PMN chemotaxis (Table 2). No signifi-

TABLE 1. *Chemotactic and glucose metabolic parameters for untreated PMN*

Parameter measured	Range of values	Mean ± SE[a]	No. of expts
Chemotaxis (PMN/10 fields)	236–4,654	1,387 ± 165	39
Glucose utilization (nmol of glucose utilized)[b]	561–1,282	896 ± 38	26
Lactic acid production (nmol of lactic acid produced)[b]	855–2,043	1,463 ± 66	26
HMP activity ($^{14}CO_2$ from [1-^{14}C]glucose, cpm)[b]	140–592	293 ± 27	25

[a] SE, Standard error.

[b] Metabolic results are expressed in the units indicated per 9×10^6 PMN in 90 min of incubation as described in Materials and Methods.

cant ($P > 0.05$) change in chemotactic responsiveness of PMN was noted at a concentration of 17.5 ml of whole tobacco smoke per 5 ml of cell suspension. However, a concentration of 35 ml of whole smoke per 5 ml of cell suspension suppressed chemotaxis to approximately 10% of the control level and was statistically significant ($P < 0.001$) as compared with controls.

The gas phase of tobacco smoke was also shown to suppress in vitro PMN chemotaxis. The gas phase of smoke at a concentration of 17.5 ml per 5 ml of cell suspension did not significantly ($P > 0.05$) affect chemotaxis. Concentrations of 35 and 52.5 ml per 5 ml of cell suspension resulted in significant ($P < 0.001$) suppression of chemotaxis to 42 and 12% of control levels, respectively.

The effects of various concentrations of WSF on PMN chemotaxis are illustrated (Fig. 1). The addition of increasing concentrations of WSF caused a progressive suppression of PMN chemotaxis. The concentration of WSF required to reduce the chemotactic responsiveness to 50% of control levels was between 25 and 50 μg of WSF per ml.

TABLE 2. *Effects of whole tobacco smoke and gas phase of smoke on PMN chemotaxis*

Treatment	Ratio of experimental to control ± SE[a]	P^b
Whole Smoke		
17.5 ml[c]	1.40 ± 0.57 (5)[d]	NS
35.0 ml	0.11 ± 0.08 (22)	<0.001
52.5 ml	0.006 ± 0.003 (4)	<0.001
Gas phase		
17.5 ml	0.96 ± 0.25 (4)	NS
35.0 ml	0.42 ± 0.06 (10)	<0.001
52.5 ml	0.12 ± 0.10 (13)	<0.001

[a] SE, Standard error.

[b] *P*, Probability that the ratio of experimental to control is equal to one. NS, Nonsignificance at the 0.05 level.

[c] Figures indicate the concentration of agent in milliliters of smoke delivered to a 5-ml suspension of PMN.

[d] Figure in parentheses, number of experiments.

Effects of whole tobacco smoke, gas phase, and WSF on glucose metabolism of PMN. Since whole smoke, gas phase, and WSF were shown to be potent inhibitors of the energy-dependent process of chemotaxis, the effects of these agents on the energy-generating mechanisms of PMN were studied (Table 3). Whole smoke and WSF, at concentrations that suppressed chemotaxis to 10% or less of that of controls, caused an approximate twofold increase over controls in glucose utilization via both the glycolytic and HMP pathways. The gas phase of tobacco smoke caused a less dramatic increase in these glucose catabolic pathways. However, the increases in glucose utilization, lactic acid production, and HMP activity were also statistically significant ($P < 0.05$) with PMN exposed to the gas phase of smoke as compared with the controls. These data suggest that tobacco smoke inhibited PMN chemotaxis

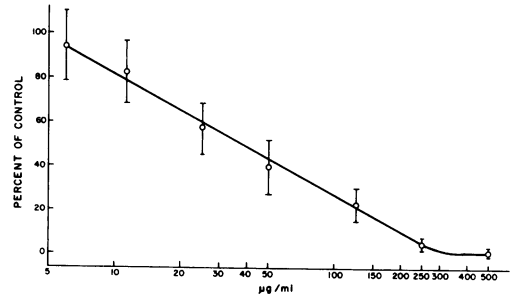

FIG. 1. *Effects of WSF of tobacco smoke on in vitro chemotaxis of PMN isolated from human peripheral blood. The results represent the mean percentage of controls (±standard error) of 6 to 10 separate experiments.*

TABLE 3. *Effects of tobacco smoke and its fractions on glucose metabolism of PMN*

Treatment	Parameter measured	Ratio of experimental to control ± SE[a]	P^b
Whole smoke (35 ml)[c]	Glucose utilized	2.08 ± 0.34 (6)[d]	<0.01
	Lactic acid produced	1.43 ± 0.12 (6)	<0.02
	HMP activity	2.53 ± 0.87 (6)	<0.05
Gas phase (52.5 ml)[c]	Glucose utilized	1.69 ± 0.21 (5)	<0.02
	Lactic acid produced	1.73 ± 0.15 (5)	<0.01
	HMP activity	2.30 ± 0.55 (5)	<0.05
WSF (250 μg/ml)	Glucose utilized	2.16 ± 0.36 (6)	<0.02
	Lactic acid produced	2.21 ± 0.54 (6)	<0.05
	HMP activity	2.29 ± 0.39 (6)	<0.02

[a, b] See table 2.

[c] Smoke concentrations are expressed in terms of smoke volume delivered to a 5-ml suspension of PMN in Gey medium.

[d] Number of experiments.

by a mechanism other than an interference with glucose metabolism. Therefore, other possible mechanisms for the inhibitory effects of tobacco smoke on PMN chemotaxis were studied.

Cytotoxic effects of tobacco smoke on PMN. PMN were incubated for 2 h at 37°C with concentrations of whole smoke (35 ml/5 ml of cell suspension), gas phase (52.5 ml/5 ml of cell suspension), and WSF (1,000 μg/ml of cell suspension). Although these concentrations suppressed the chemotactic response to less than 10% of that of the controls, the viability of the PMN was 98% or greater as determined by trypan blue exclusion. Quantitation of the number of PMN per milliliter of suspension demonstrated the same cellular concentrations in suspensions incubated in the presence and absence of these substances as were present in the original suspension. Similar results were obtained when tobacco smoke-treated PMN suspensions were exposed to fluorescein diacetate with ethidium bromide as a counterstain for dead cells.

The effect of WSF on cellular integrity was tested by exposure of PMN to this agent for 2 h and assaying the supernatant fluid after centrifugation for cytoplasmic LDH or lysosomal β-glucuronidase (Table 4). No measurable LDH was released from PMN after exposure to a concentration (1,000 μg/ml) of WSF in excess of that necessary to suppress chemotaxis. WSF at concentrations of 250 to 1,000 μg/ml also did not cause a greater release of lysosomal β-glucuronidase than was observed with untreated cells. However, WSF at a concentration of 5,000 μg/ml gave a twofold increase in β-glucuronidase release over that of controls. However, this increased activity still represented less than 10% of that obtained with broken-cell suspensions. Neither the LDH nor the β-glucuronidase activities of broken-PMN suspensions were affected by incubation for 2 h with WSF concentrations as great as 5,000 μg/ml.

Other effects of tobacco smoke. The effects of tobacco smoke and its fractions on chemotaxis cannot be attributed to changes in pH, since the addition of these substances did not appreciably alter the final pH of the PMN suspension (pH 7.3 ± 0.1).

Previous exposure of PMN to a chemotactic agent prevents these cells from responding to another chemotactic challenge (30). Thus, a possible explanation for the inhibitory effects of tobacco smoke on PMN chemotaxis is that these substances act as chemotactic agents. The WSF was shown not to act as a chemotactic agent over a wide range of concentrations (125 to 5,000 μg/ml).

TABLE 4. *Effects of WSF of tobacco smoke on cellular integrity of PMN*

Treatment	β-Glucuronidase activity (U/18 h per 3 × 10⁶ PMN)	LDH activity (U/3 × 10⁶ PMN)
None	0.020 ± 0.001[a]	ND[b]
WSF (μg/ml)		
250	0.019 ± 0.002	ND
500	0.020 ± 0.002	ND
1,000	0.021 ± 0.002	ND
5,000	0.038 ± 0.005	ND
Sonicated PMN	0.510 ± 0.003	0.054 ± 0.001
Sonicated PMN + WSF (5,000 μg/ml)	0.506 ± 0.004	0.056 ± 0.001

[a] Results are expressed as means ± standard error of six to eight separate experiments.
[b] ND, The enzyme activity was not detectable.

Tobacco smoke and its fractions have been tested for their ability to inhibit random migration as well as the directional migration of chemotaxis. These test agents at concentrations that inhibited directional migration were shown to be equally effective as inhibitors of random migration.

Finally, whole smoke, gas phase of smoke, and WSF were also tested for their effect on activated, autologous serum. Endotoxin-activated serum was incubated with inhibitory levels of whole smoke (35 ml/5 ml), gas phase (52.5 ml/5 ml), or WSF (1,000 μg/ml) for 2 h at 37°C and dialyzed overnight at 4°C against Gey medium. The tobacco smoke-treated, activated serum was equally effective as a chemotactic agent when compared with untreated activated serum.

Protective action of cysteine against tobacco smoke inhibition of chemotaxis. Cysteine provides some protection to PMN against chemotactic inhibition by tobacco smoke products (Table 5). Cysteine (10 mM), added before tobacco smoke exposure, afforded limited protection to PMN against the effects of whole tobacco smoke and the gas phase of tobacco smoke (at concentrations that completely inhibited chemotaxis). The inhibition of chemotaxis by WSF (1,000 μg/ml) was completely prevented by the prior addition of cysteine. To determine whether the effects of prior exposure to WSF were reversed by cysteine, PMN were exposed to WSF (in the absence of cysteine), collected by centrifugation, resuspended in an equivalent volume of Gey medium in the presence and absence of cysteine, and tested for chemotactic response. The inhibition of PMN chemotaxis by WSF was only slightly reversed by the removal of agents present in solution. However, the remaining inhibition could not be overcome, even partially, by the addition of cysteine.

TABLE 5. *Protective action of cysteine against tobacco smoke inhibition of chemotaxis*

Treatment	10 mM cysteine added[a]	Ratio of experimental to control ± SE[b]
None	+	1.02 ± 0.14
Whole smoke[c]	−	0 ± 0
Whole smoke[c]	+	0.46 ± 0.14
Gas phase[c]	−	0 ± 0
Gas phase[c]	+	0.67 ± 0.14
WSF (1,000 μg/ml)	−	0.002 ± 0.001
WSF (1,000 μg/ml)	+	0.96 ± 0.23
WSF (1,000 μg/ml)[d]	−	0.25 ± 0.12
WSF (1,000 μg/ml)[d]	+	0.20 ± 0.02

[a] Unless otherwise indicated, cysteine was added to PMN suspension prior to the addition of tobacco smoke products.

[b] Ratios are expressed as the mean ± standard error (SE) of three experiments.

[c] PMN were exposed to whole tobacco smoke and the gas phase of tobacco smoke at concentrations of 35 and 52.5 ml per 5 ml of cell suspension, respectively.

[d] PMN were exposed to WSF (in the absence of cysteine), collected by centrifugation, resuspended in an equivalent volume of Gey medium in the presence and absence of cysteine, and tested for their chemotactic responsiveness.

DISCUSSION

The results of this study indicate that whole tobacco smoke, gas phase of smoke and WSF are potent inhibitors of PMN chemotaxis. Thus, in vitro PMN chemotaxis serves as a sensitive, biological system for the study of the deleterious agents of tobacco smoke. WSF at a concentration of 250 μg/ml suppressed in vitro PMN chemotaxis to 10% of that of controls. This level of inhibition was achieved with 35 ml of whole tobacco smoke per 5 ml of PMN suspension. Although whole tobacco smoke was a more potent inhibitor than was gas phase of smoke, the gas phase of smoke retained a considerable portion of the inhibitory capacity of whole smoke. These data suggest that the agents inhibitory to PMN chemotaxis were associated with both the particulate and gas-phase fractions of whole tobacco smoke.

This study has also attempted to determine the physiological mechanism of PMN which is most deleteriously affected by tobacco smoke and which accounts for the observed inhibition of chemotaxis. Little information exists concerning the biochemical and energetic mechanisms required for PMN chemotaxis. However, the directed migration of PMN in response to specific chemotactic factors has been shown to be markedly suppressed by inhibitors of the glucose metabolic pathways (9, 19). Further, the interaction of human PMN with purified chemotactic factor was shown to be accompanied by an increased rate of glucose catabolism via both the glycolytic and HMP pathways, and metabolic inhibitors of either pathway resulted in a suppression of chemotaxis (16). Since the stimulation of glucose metabolism in PMN was associated with a maximal chemotactic response, the effects of tobacco smoke on glucose metabolism of PMN were studied.

Concentrations of whole smoke and WSF that suppressed PMN chemotaxis were shown to cause a twofold increase in the glucose catabolic pathways (i.e., both glycolysis and the HMP shunt). Gas phase of smoke also caused a significant (1.7-fold) increase in the rates of these glucose catabolic pathways. Part of these increases in glucose metabolism may result from phagocytosis, especially in those tobacco smoke fractions containing particulate matter (i.e., whole smoke and the WSF), since increased rates of both pathways have been demonstrated in PMN upon phagocytosis of killed bacteria (11). However, phagocytosis does not account for the increased glucose catabolism observed with gas phase of smoke that lacks particulate matter.

PMN were found to utilize oxidative metabolism via the tricarboxylic acid cycle to a minimal extent. The extent of glucose metabolism via oxidative metabolism is consistent with the observations that PMN contain few mitochondria and that resting cells have a low capacity for metabolism via this pathway (19). Tobacco smoke or its fractions had no measurable effect on glucose metabolism via the tricarboxylic acid cycle as indicated by $^{14}CO_2$ release from [6-^{14}C]glucose.

Increased glucose metabolism of PMN via the glycolytic and HMP pathways in response to exposure to tobacco smoke was an unexpected result. These data suggest that PMN exposed to tobacco smoke have a higher resting metabolism and thus higher energy requirement than do untreated cells. The increased glucose metabolism of PMN exposed to tobacco smoke in vitro may also occur with exposure in vivo. Anderson et al. (2) noted that PMN from peripheral blood of heavy cigarette smokers frequently give a false-positive nitroblue tetrazolium test. This test serves as an indicator of reduced pyridine nucleotide, and thus of the glucose catabolic rate (27). The metabolic experiments in the present study suggest that there is no direct correlation between the effects of tobacco smoke on glucose metabolism and PMN chemotaxis. However, the effects of tobacco smoke on glucose metabolism may rep-

resent a secondary effect resulting from the primary mechanism of chemotactic inhibition by tobacco smoke.

The effect of tobacco smoke on PMN chemotaxis and glucose metabolism has significance in relation to the biochemical mechanisms of PMN chemotaxis. The fact that tobacco smoke inhibits chemotaxis while stimulating glucose metabolism supports the earlier hypothesis that the stimulation of glucose metabolism is not alone sufficient for optimal chemotaxis (16).

Further attempts were made to delineate the primary mechanism whereby tobacco smoke inhibited PMN chemotaxis. Whole tobacco smoke, the gas phase of tobacco smoke, and WSF, at concentrations in excess of those required to inhibit chemotaxis, have been shown to have no cytotoxic effects on PMN as measured by trypan blue exclusion (>98% viability). This conclusion was supported by viability measurements using fluorescein diacetate and ethidium bromide. WSF was further shown to have no effect on PMN integrity as measured by the release of cytoplasmic LDH or lysosomal β-glucuronidase activities. The viability of the PMN treated with WSF was also supported by the dose-response curve generated for this agent.

The results of this study suggest that the effects of tobacco smoke on PMN chemotaxis result from a direct interaction of these deleterious agents with the cell. Tobacco smoke did not affect the chemotactic agent since activated, autologous serum, treated with inhibitory concentrations of tobacco smoke fractions and subsequently dialyzed, was shown to be equally effective as a chemotactic stimulus as untreated activated serum. A direct effect of tobacco smoke on the target cell is also suggested by the fact that whole smoke, the gas phase of smoke, and WSF at concentrations that inhibited the directional migration of chemotaxis also inhibited random migration. Additional evidence in support of this mechanism is the fact that chemotactic inhibition by WSF was prevented by cysteine. However, cysteine afforded only partial protection to PMN chemotaxis against inhibition by whole smoke and the gas phase of smoke. The observed differences in the protection afforded by cysteine against whole smoke and the gas phase of smoke versus WSF may depend on the method of delivery of tobacco smoke products. Direct delivery of whole smoke or gas phase of smoke to PMN suspensions allows for the interaction of the cells and volatile components that were lost from the WSF. Further experiments indicated that the inhibiton of PMN chemotaxis by WSF exposure (in the absence of cysteine) could not be reversed by the later addition of cysteine. Thus, the major inhibitory effects of tobacco smoke on PMN chemotaxis result from irreversible reactions, possibly between the sulfhydryl groups of cellular proteins and the agents of tobacco smoke. In support of this hypothesis, tobacco smoke has been shown to contain unsaturated aldehydes (e.g., acrolein and crotonaldehyde) (24), which are thiol-alkylating reagents (12). Further, a similar thiol-alkylating agent, iodoacetate, is a potent inhibitor of PMN chemotaxis (9). However, the mechanism whereby iodoacetate inhibits chemotaxis may be distinctly different from that caused by tobacco smoke, since iodoacetate causes a suppression of both the HMP and glycolytic stimulation by chemotactic factor (16).

The limited recovery (20 to 25%) of PMN chemotactic responsiveness observed after the removal of excess agents in solution suggest that some of the inhibitory effects of WSF are attributable to reversible inhibitors. Thus, the total inhibitory capacity of tobacco smoke may result from two or more different mechanisms and/or types of compounds.

Among other possible interactions between tobacco smoke and PMN that could account for the observed inhibition of chemotaxis are: the generation of a neutrophil-immobilizing factor from leukocytes, and the deactivation of PMN by prior exposure to a chemotactic factor.

A neutrophil-immobilizing factor derived from leukocytes by acid incubation, by endotoxin treatment, or as a result of phagocytosis has been described (15). It is unlikely that any of these conditions prevailed with exposure of PMN to tobacco smoke, especially the gas phase of smoke.Therefore, an immobilizing factor can probably be discounted as the cause for the observed effects of smoke on PMN chemotaxis. However, an immobilizing factor generated by chemical insult to the cell cannot be rigorously excluded.

The deactivation of rabbit neutrophils to chemotactic stimulus has been produced by prior exposure of these cells to a chemotactic agent (30). The fact that WSF did not act as a chemotactic agent suggests that the observed inhibition of chemotaxis by WSF was not a result of deactivation of PMN in this manner.

The effects of tobacco smoke on the two phagocytic cell types, the polymorphonuclear leukocyte and the pulmonary macrophage, have been studied. In contrast to the effects of tobacco smoke on PMN chemotaxis reported in this study, both the chemotactic responsiveness and the random migration of pulmonary macro-

phages from smokers were greater than those of macrophages from nonsmokers (31). Thus, the chemotactic mechanism of these two phagocytic cell types may differ in their susceptibility to tobacco smoke products. In comparison to the PMN, the pulmonary macrophage depends to a greater extent on oxidative metabolism for energy generation (26). Despite these differences in metabolism, the pulmonary macrophage, like the PMN, when exposed to tobacco smoke exhibits a threefold increase in glucose catabolic rate (18).

In the present study, the chemotaxis of PMN exposed in vitro to tobacco smoke or its fractions was markedly suppressed. Previous studies have also indicated that the chemotactic responsiveness of PMN isolated from the peripheral blood of smokers was depressed as compared with PMN isolated from blood of either nonsmokers or abstaining smokers (25). The results of our studies suggest a direct effect of tobacco smoke on PMN with in vitro exposure techniques. However, it remains to be established whether the concentrations of tobacco smoke constituents (or their metabolites) in the blood or PMN of smokers can attain sufficient levels to directly inhibit chemotaxis or whether another mechanism may be operative.

PMN chemotaxis might be expected to be inhibited in those tissues exposed directly to tobacco smoke. Thus, the chemotaxis of PMN might be severely affected in the oral cavity and the lung. In support of this hypothesis, the WSF of tobacco smoke has been shown to cause a depression in the gingival PMN migration rate when applied to the gingiva of beagle dogs (unpublished observations).

The in vitro effects of this study cannot be equated with effects in vivo. However, these in vitro methods are being used to determine the components of tobacco smoke most deleterious to PMN function. Further, studies are in progress to determine the effects of tobacco smoke on PMN chemotaxis with the aim of elucidating the inhibitory mechanism involved.

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