Effects of Cigarette Smoke and Its Constituents on the Adherence of Polymorphonuclear Leukocytes

RAYMOND B. BRIDGES,^{1*} LILY HSIEH,¹ AND DENNIS G. HAACK²

Department of Oral Biology, College of Dentistry, University of Kentucky, Lexington, Kentucky 40536,¹ and Biostatistics and Epidemiology Section, Tobacco and Health Research Institute, University of Kentucky, Lexington, Kentucky 40506²

The *in vitro* effects of the water-soluble fraction of whole cigarette smoke (WSF) and two α,β -unsaturated aldehydes of cigarette smoke (acrolein and crotonaldehyde) on polymorphonuclear leukocyte (PMNL) adherence were determined with nylon fiber columns. Each of these cigarette smoke constituents caused a dose-dependent inhibition of PMNL adherence. However, at least fivefold higher concentrations of these agents were necessary to inhibit adherence as compared with those necessary to achieve the same level of inhibition of PMNL chemotaxis. Furthermore, inhibition of adherence by WSF could be differentiated from its effects on chemotaxis in that reduced glutathione completely protected chemotaxis from the effects of WSF but only afforded partial protection to PMNL adherence. These data suggest that the inhibitory effects of WSF, acrolein, and crotonaldehyde on PMNL chemotaxis are not due to their inhibition of adherence. Finally, although PMNL adherence is considered to be an integral part of the chemotactic mechanism, differentiation between these two PMNL functions may be possible, since some inhibitors of chemotaxis do not have corresponding inhibitory effects on adherence.

Polymorphonuclear leukocytes (PMNL) play an important role in host defense against bacterial infections. It has been suggested that the *in vivo* effects of cigarette smoking on peripheral blood PMNL may contribute to the increased susceptibility of smokers to infections (7). The specific functions of peripheral blood PMNL which have been shown to be deleteriously affected by smoking include random migration (7) and chemotaxis (15); however, the bactericidal capacity of whole blood was unaffected by smoking (7, 15). The *in vivo* mechanism whereby smoking affects PMNL migration (i.e., random or directed) is not known.

In vitro studies of the effects of cigarette smoke on PMNL motility demonstrated that whole cigarette smoke, its gas phase, or the water-soluble fraction of cigarette smoke (WSF) were potent inhibitors of both random migration and chemotaxis of PMNL (3). These effects on motility were at least partially due to the direct effect of oxidants or thiol-reactive components such as the α,β -unsaturated aldehydes of cigarette smoke, acrolein and crotonaldehyde (4). In support of this conclusion, sulfhydryl-reducing agents completely prevented the *in vitro* inhibition of WSF, acrolein, or crotonaldehyde on chemotaxis (3, 4). However, at concentrations inhibitory to motility, WSF, acrolein, or crotonaldehyde stimulated carbohydrate metabolism twofold via both the glycolytic and the hexose-

monophosphate pathways. Therefore, the *in vitro* inhibitory effects of these agents on motility could not be correlated with their effects on carbohydrate metabolism.

Because PMNL chemotaxis is dependent upon adherence to a substratum (11, 18), the present study sought to determine whether the effects of cigarette smoke constituents on chemotaxis were due to an inhibition of chemotaxis *per se* or to effects on adherence. In this communication, we show that the previously demonstrated effects of WSF, acrolein, or crotonaldehyde on chemotaxis (3, 4) are not due to the effects of these agents on adherence.

MATERIALS AND METHODS

Isolation of PMNL. PMNL were isolated from the peripheral blood of nonsmoking, healthy male volunteers ranging in age from 20 to 40 years. This study was approved by the Human Investigations and Studies Committee of the University of Kentucky. Venous blood (140 ml) was drawn at 8 a.m. PMNL were obtained by dextran (average molecular weight, 234,000) sedimentation of heparinized whole blood (6), separation of granulocytes from mononuclear cells by the Hypaque-Ficoll technique (2), and hypotonic saline lysis of residual erythrocytes (6). Cells were resuspended in Gey balanced salt solution containing 2% (wt/vol) bovine serum albumin. Cell counts were performed with Petroff-Hauser counting chambers. The PMNL suspensions were adjusted to 1.5×10^7 PMNL/ml by the addition of Gey medium. This procedure

produced PMNL populations of greater than 98% purity with greater than 98% viability as measured by trypan blue exclusion (4).

Assay for PMNL adherence. Although a number of assay systems exist for the determination of PMNL adherence (1, 10, 13, 16, 17), a modification of the nylon fiber column method was chosen because of its simplicity and reproducibility (14). Purified PMNL suspensions in Gey medium containing 2% (wt/vol) bovine serum albumin were used instead of whole blood. Purified PMNL suspensions were used so that the effect of the test agents on PMNL adherence might be directly compared with the previously observed *in vitro* effects of these agents on PMNL chemotaxis (3, 4).

Nylon fiber columns were prepared with disposable 14.6-cm Pasteur pipettes. The pipette tips were heated and then drawn out to create an aperture just large enough to admit the shaft of a 22-gauge needle. Two lines (15 ± 0.5 mm apart) were marked on the pipettes beginning at the midpoint of the pipette shoulder to the predetermined top of the packed nylon fiber bed. The standardized pipettes were then packed with scrubbed nylon fiber (3 denier, 3.81 cm) so that the nylon fiber bed was exactly 15 mm long. The columns were clamped vertically and placed in a 37°C incubator with the tips of the columns extending into the top of small plastic tubes (12 by 75 mm).

Two types of experiments were performed: (i) with varying weights of nylon fiber with constant amounts of inhibitor or (ii) with columns of constant nylon fiber weight (80 mg) while varying inhibitor concentrations. These latter type of experiments were done to demonstrate the dose-dependent manner by which the inhibitory agent decreased PMNL adherence. An 80-mg amount of nylon fiber was used for these columns, since this weight of nylon fiber was found to trap a majority of PMNL ($80.7 \pm 1.8\%$ adherence in 26 experiments), yet the percentage of adherent PMNL was very sensitive to inhibitory agents.

PMNL suspensions (1 ml) were warmed to 37°C in a water bath for 15 min, mixed in a Vortex mixer, and applied to the top of a nylon fiber column with a siliconized pipette. Samples were allowed to filter through the columns for 5 to 10 min. The PMNL in the effluent were counted, and the percentage of PMNL adhering to the column was calculated by using the following equation: percent PMNL adherence = $100 - [(PMNL \text{ per milliliter in the effluent sample} / PMNL \text{ per milliliter in the original sample}) \times 100]$. The PMNL adherence was defined as the mean value obtained with three columns.

Cigarette smoke fraction. Fresh WSF was obtained from John Benner of the University of Kentucky Tobacco and Health Research Institute. The WSF was prepared as previously described (3) except that 2R1 reference cigarettes of the University of Kentucky Tobacco and Health Research Institute were used instead of the 1R1. Stock WSFs had a residue concentration of 50 mg/ml. Dilutions of WSF were made in Gey medium, and the final concentration of WSF was expressed as milligrams of residue per milliliter of cell suspensions.

Other inhibitors of adherence. Two unsaturated aldehydes of cigarette smoke, acrolein (2-propenal)

and crotonaldehyde (2-butenal), previously shown to cause ciliastasis (20) and inhibition of PMNL chemotaxis (4), were tested as inhibitors of PMNL adherence. These inhibitors were freshly prepared in Gey medium, adjusted to pH 7.4, and added to PMNL suspensions to give the final concentrations indicated. An equivalent amount of Gey medium was added to control PMNL suspensions. The concentrations of inhibitors used did not affect PMNL viability as measured by trypan blue exclusion (19). PMNL suspensions were preincubated with inhibitors in a 37°C water bath for 15 min before their application to a nylon fiber column.

A sulfhydryl group inactivator, *p*-hydroxymercuribenzoate, was also tested as an inhibitor of PMNL adherence. A 20 mM stock solution prepared in 0.1 N NaOH was used. To maintain the pH of the PMNL suspension, the stock was diluted in 0.1 N NaOH, and 1 μ l was added to 1 ml of PMNL suspension to give the final concentrations indicated.

Reduced glutathione, which protects PMNL chemotaxis from inhibition by WSF (R. B. Bridges and L. J. T. Huang, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, E205, p. 115), was tested to determine whether it would also protect PMNL adherence from inhibition by WSF or acrolein. Reduced glutathione (final concentration, 5 mM) was added to PMNL suspensions before the addition of inhibitor. This mixture was then incubated with the inhibitor under test for 15 min in a 37°C water bath before the adherence assay was performed.

Statistical analyses. Data were analyzed by analysis of variance. The means $\pm$ the standard error of the experimental data were plotted in the graphs. The curves in these graphs represent the best least-square quadratic fit of the experimental points.

Chemicals. Clinical grade dextran (average molecular weight, 234,000), ficoll (dialyzed and lyophilized; molecular weight, 400,000), *p*-chloromercuribenzoic acid, and reduced glutathione were all products of Sigma Chemical Co., St. Louis, Mo. Hypaque (sodium, 50%) was a product of Winthrop Laboratories, Division of Sterling Drug Inc., New York, N.Y. Gey media containing 2% (wt/vol) bovine serum albumin was obtained from Flow Laboratories, Inc., McLean, Va. Finally, scrubbed nylon fiber (3 denier, 3.81 cm, type 200) was obtained from Fenwal Laboratories, Division of Travenol Laboratories, Inc., Deerfield, Ill.

RESULTS

Effects of WSF on PMNL adherence as a function of nylon fiber weight. The percentages of control or WSF-treated PMNL adhering to columns containing various weights of nylon fiber are shown in Fig. 1. Curves of PMNL adherence were obtained by varying the nylon fiber weight from 50 to 150 mg. The presence of WSF at a concentration of 0.5 mg of residue per ml significantly reduced ($P < 0.002$) PMNL adherence. In the absence of WSF, PMNL adherence increased from 58.0 to 98.1% at 50 and 150 mg of nylon fiber, respectively. In the presence of WSF, PMNL adherence increased from 35.5

FIG. 1. Percentage of PMNL adherence at various weights of nylon fiber and the effects of WSF. Symbols: ●, no addition; ×, 0.5 mg of residue per ml of WSF. Data points represent the mean $\pm$ standard error of three determinations. The curves represent the best least-square fit of the data.

to 91.4% at 50 and 150 mg of nylon fiber, respectively. Thus, a concentration of WSF (0.5 mg of residue per ml) which had previously been shown to inhibit PMNL chemotaxis by more than 90% without affecting cellular viability (3) was also shown to significantly inhibit PMNL adherence.

Effects of WSF concentration on PMNL adherence and the protection afforded by reduced glutathione. The dose-dependent inhibition of PMNL adherence by WSF is shown in Fig. 2. In the absence of reduced glutathione, PMNL adherence decreased from 94% (WSF = 0) to 41% (WSF = 1 mg of residue per ml). Increasing the WSF concentration resulted in a progressive and significant ($P < 0.0001$) decrease in PMNL adherence. Furthermore, the decrease in PMNL adherence observed in the presence of WSF alone was significantly ($P < 0.003$) greater than that observed in PMNL suspensions containing both 5 mM reduced glutathione and WSF. Thus, reduced glutathione provided partial protection from the inhibitory effects of WSF. Reduced glutathione in the absence of WSF did not affect the adherence of PMNL ($P > 0.4$).

Effects of acrolein on PMNL adherence and the protection afforded by reduced glutathione. The effects of various concentrations

of acrolein on adherence with 80 mg of nylon columns are shown in Fig. 3. The addition of increasing concentrations of acrolein to PMNL suspensions resulted in a progressive reduction in adherence ($P < 0.0001$). This reduction in adherence was greater in the absence of 5 mM reduced glutathione than in the presence of this protector ($P < 0.0001$). As with WSF, reduced glutathione only partially protected PMNL adherence from acrolein inhibition. Also, the viability of PMNL treated with the highest concentration of acrolein (150 μ M) was greater than 93% as determined by trypan blue exclusion.

Effects of crotonaldehyde on PMNL adherence. The dose-dependent inhibition of PMNL adherence by crotonaldehyde is shown in Fig. 4. As with acrolein, increasing concentrations of crotonaldehyde resulted in a significant ($P < 0.0001$) and progressive decrease in PMNL adherence. However, crotonaldehyde had no effect on PMNL viability at the highest concentration tested (250 μ m).

Effects of *p*-hydroxymercuribenzoate on

FIG. 2. Effects of WSF on PMNL adherence at constant weight of nylon fiber (80 mg) and the protective effects of reduced glutathione. Symbols: ●, WSF added to give final concentrations indicated; ×, reduced glutathione (5 mM) and WSF added. Data points represent the mean $\pm$ standard error of three determinations. The curves represent the best least-square fit of the data.

FIG. 3. Effects of acrolein on PMNL adherence at constant weight of nylon fiber (80 mg) and the protective effects of reduced glutathione. Symbols: ●, acrolein added to give final concentrations indicated; x, reduced glutathione (5 mM) and acrolein added. Data points represent the mean $\pm$ standard error of three determinations. The curves represent the best least-square fit of the data.

PMNL adherence. The sulfhydryl group inactivator, *p*-hydroxymercuribenzoate, was a potent inhibitor of PMNL adherence (Fig. 5). At concentrations between 3 and 6 μ M, this compound significantly ($P < 0.002$) inhibited adherence but did not affect viability.

DISCUSSION

The purpose of the present study was to determine whether WSF, acrolein, or crotonaldehyde inhibited PMNL adherence. Furthermore, because adherence is an integral part of the chemotactic mechanism (11, 18), this investigation sought to determine whether the effects of these cigarette smoke components on adherence could be correlated with their previously reported inhibitory effects on chemotaxis (3, 4). This study suggests that although WSF, acrolein and crotonaldehyde inhibited PMNL adherence, their effects on adherence and chemotaxis differed. First, these cigarette smoke components were more potent inhibitors of chemotaxis (3, 4) than adherence (Fig. 2, 3, and 4). Second, although the inhibitory effects of WSF, acrolein, or crotonaldehyde on PMNL chemotaxis were apparently due to their thiol-inactivating properties (3, 4), their effects on PMNL adherence

appear to be primarily due to other mechanisms.

WSF (Fig. 2), acrolein (Fig. 3), and crotonaldehyde (Fig. 4) inhibited PMNL adherence in a dose-dependent manner. The inhibition of PMNL adherence by WSF, acrolein, or crotonaldehyde appeared not to be solely due to the thiol-inactivating properties of these compounds, since reduced glutathione, a sulfhydryl reducing agent, afforded significant but limited protection to adherence from WSF and acrolein inhibition (Fig. 2 and 3). Furthermore, *p*-hydroxymercuribenzoate (Fig. 5), a thiol inactivator, was a potent inhibitor of adherence; however, the dose-response curve obtained with this inhibitor was different from those obtained with either WSF (Fig. 2), acrolein (Fig. 3), or crotonaldehyde (Fig. 4). These data suggest that although thiol inactivators are potent inhibitors of PMNL adherence, the inhibitory effects of WSF, acrolein, or crotonaldehyde on adherence were apparently not attributable to thiol inactivation.

Since PMNL adherence is thought to be an essential event for chemotaxis (11, 18), the effects of these cigarette smoke components on PMNL adherence and chemotaxis were com-

FIG. 4. Effects of crotonaldehyde on PMNL adherence at constant weight of nylon fiber (80 mg). Symbol: ●, crotonaldehyde added to give the final concentration indicated. Data points represent the mean $\pm$ standard error of three determinations. The curves represent the best least-square fit of the data.

FIG. 5. Effects of *p*-hydroxymercuribenzoate on PMNL adherence at constant weight of nylon fiber (80 mg). Symbol: ●, *p*-Hydroxymercuribenzoate was added to give the final concentrations indicated. Data points represent the mean $\pm$ standard error of three determinations. The curves represent the best least-square fit of the data.

pared. These comparisons were made to determine whether the effects of cigarette smoke components on PMNL chemotaxis could be attributed to their effects on adherence. Comparisons of WSF, acrolein, and crotonaldehyde concentrations necessary to cause 50% inhibition of PMNL adherence or chemotaxis are shown in Table 1. The concentration of each agent causing 50% inhibition of adherence was calculated by the appropriate least-square fit quadratic equation which described the dose-dependent inhibition of PMNL adherence. The best-fit quadratic equations in each case could account for greater than 94% of the experimental variation. By these comparisons, PMNL adherence was 5 to 15 times less sensitive to inhibition by WSF, acrolein, or crotonaldehyde than was chemotaxis.

The greater sensitivity of chemotaxis to inhibition by these agents is further illustrated by the percentage inhibition of adherence which occurs at a concentration of agent inhibiting chemotaxis by 50% (Table 1). Concentrations of WSF, acrolein, or crotonaldehyde inhibiting chemotaxis by 50% resulted in inhibition of adherence by 4.9, 7.8, or 15.4%, respectively. Thus, PMNL chemotaxis is much more sensitive than

adherence to inhibition by WSF, acrolein, or crotonaldehyde.

Parenthetically, nicotine, the most powerful pharmacological agent in cigarette smoke, had no statistically significant effect on PMNL adherence in concentrations as great as 3 mM (data not shown). Nicotine at this concentration was previously shown to inhibit chemotaxis by 50% (4).

In addition to differences in sensitivity of adherence and chemotaxis to inhibition by cigarette smoke constituents, the inhibitory mechanisms also may differ for these PMNL functions. Reduced glutathione afforded significant yet incomplete protection to PMNL adherence, whereas cysteine (3, 5) or reduced glutathione (R. Bridges and L. J. T. Huang, Abstr. Annu. Meet. Am. Soc. Microbiol., 1977, E205, p. 115) completely protects chemotaxis from these inhibitors. These data support the previous suggestion that cigarette smoke components inhibit PMNL adherence by mechanism(s) in addition to thiol inactivation, whereas their inhibition of chemotaxis is due to thiol-inactivation (3, 5). However, one cannot exclude the possibility that these differences in protection afforded to adherence and chemotaxis by sulfhydryl reducing agents may result from the differential sensitivity of these PMNL functions to inhibition by cigarette smoke components or the different assays utilized to measure these functions or both.

Thus, PMNL chemotaxis and adherence were observed to differ in relation to both sensitivity to inhibition by cigarette smoke components and the protection afforded by sulfhydryl reducing agents. These observations support the conclusion that the inhibitory effects of cigarette smoke components on PMNL chemotaxis are not due to their effects on adherence. Furthermore, al-

TABLE 1. Concentrations of cigarette smoke components necessary to achieve 50% inhibition

PMNL function	Cigarette smoke component		
	WSF (mg of residue/ml)	Acrolein (μM)	Crotonaldehyde (μM)
Chemotaxis ^a	0.050	15	40
Adherence ^b	0.745 (4.9)	121.5 (7.8)	218 (15.4)

^a Concentrations of WSF, acrolein, or crotonaldehyde necessary for 50% inhibition of chemotaxis as previously described (3, 4).

^b Doses required to produce 50% inhibition of adherence were calculated with the best-fit quadratic equations obtained from the dose-response curves. The calculated percentage of inhibition of PMNL adherence for the component, at a concentration which produced 50% inhibition of chemotaxis, is given in parenthesis.

though PMNL adherence is essential for chemotaxis (11, 18), differentiation between these two PMNL functions may be possible since some chemical inhibitors of chemotaxis do not have corresponding inhibitory effects on adherence.

Although WSF, acrolein, and crotonaldehyde inhibit PMNL adherence, these relatively high, inhibitory concentrations are probably never attained in the peripheral blood of smokers. However, inhibition of peripheral blood PMNL adherence as a direct or indirect consequence of smoking might explain the relative granulocytosis which occurs in smokers (8, 9). The intensity of granulocyte adherence has been postulated to play an integral role in granulocyte movement between the various body compartments and thus to affect granulocyte kinetics (12).

If cigarette smoke affects adherence of peripheral blood PMNL, this effect, as well as smoking effects on PMNL motility (7, 15), could greatly diminish the delivery of PMNL to the site of infection and, thus, diminish host resistance to bacterial infections. To determine the effects of smoking on PMNL function, an epidemiological study is underway in our laboratory to determine the number, motility, and adherence of peripheral blood PMNL in smoking and non-smoking populations.

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