Neutrophil Priming by Cigarette Smoke Condensate and a Tobacco Anti-Idiotypic Antibody

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A polyphenol-rich reagent, referred to as CSC, was isolated from cigarette smoke condensate and shown to prime purified human neutrophils. A mouse monoclonal anti-idiotypic antibody directed against the polyphenol-reactive determinants on a rabbit polyclonal anti-tobacco glycoprotein antibody was generated and shown to also prime neutrophils. After priming by CSC or tobacco anti-idiotypic antibody, there was a 2.5-fold to threefold increase in CD11b/18 expression and doubling of the number of formyl-methionyl-leucyl-phenylalanine receptors on the cells. The primed cells showed a twofold increase, compared with unprimed cells, in production of superoxide and release of neutrophil elastase after stimulation with formyl-methionyl-leucyl-phenylalanine. Neutrophils in peripheral blood of cigarette smokers have been shown to be primed and more responsive to activating agents. The priming effects attributed to whole cigarette smoke have been demonstrated in these studies using purified neutrophils and CSC or tobacco anti-idiotypic antibody. These studies are a first step in testing the hypothesis that the inflammatory process contributing to progression of chronic obstructive pulmonary disease in ex-smokers may be driven, in part, by tobacco anti-idiotypic antibodies. This hypothesis is novel and carries with it the implication of a heretofore unrecognized autoimmune component in the disease process manifested through production of anti-idiotypic antibodies with tobacco-like activity. (*Am J Pathol* 2000, 157:1735–1743)

Cigarette smoking is a major risk factor for a number of diseases including chronic obstructive pulmonary disease (COPD), arteriosclerosis, cardiovascular disease, cerebrovascular disease, and Crohn's disease. Inflammation is common to the pathogenesis of these disorders, yet the mechanisms underlying the role of smoking in these diseases are primarily unknown. Recent studies have shown that neutrophils isolated from the peripheral blood of smokers and individuals exposed to second hand smoke are primed, meaning that on challenge with formyl-methionyl-leucyl-phenylalanine (fMLP) their neu-

trophil oxidative burst response, and in one study release of neutrophil elastase, is greatly enhanced.^{1–3} Therefore, in smokers, primed neutrophils may function to amplify and prolong inflammation thereby promoting sustained tissue injury. Events that ordinarily would not lead to development of disease may be more likely to occur because of the enhanced response of primed cells after activation.

We have demonstrated *in vitro* the priming effects of *in vivo* exposure to cigarette smoke using neutrophils purified from whole blood of nonsmokers and a low molecular weight polyphenol-rich fraction, referred to as cigarette smoke condensate (CSC), extracted from cigarette smoke.⁴ In cigarette smoking-associated diseases, smoking cessation should remove the priming agent thereby reducing inflammation and slowing the disease process. This, however, is not always the case. In some patients with COPD the disease continues long after smoking has stopped.⁵ Airway inflammation, marked by a significant increase in the percentage of neutrophils in the sputum and cells containing IL-8, has been demonstrated in COPD patients at least 1 year after smoking cessation.⁶ One hypothesis to explain ongoing inflammation in these patients would be the presence of anti-idiotypic antibodies with activity similar to agents in tobacco, like CSC, that could function to continuously prime neutrophils and thereby contribute to the continuation of the inflammatory process. To develop an *in vitro* model to test this hypothesis, we generated a mouse monoclonal tobacco anti-idiotypic antibody (TAIA) reactive with rabbit antibodies to polyphenols in tobacco that is functionally similar to CSC.⁷ Both CSC and TAIA prime neutrophils to show a significant increase in fMLP receptors and expression of the integrin CD11b/18 on the cell membrane. Neutrophils primed with CSC and TAIA also have significantly increased oxidative burst activity and release of elastase after stimulation with fMLP compared with unprimed controls. Neutrophil priming by CSC is abrogated in the presence of SB203580, an inhibitor of MAPK p38 activation and phosphorylation, indicating a role for activation of MAPK pathways in this process.

The results presented in this *in vitro* study support the hypothesis that TAIA, which is functionally similar to CSC,

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may contribute to driving progression of COPD long after smoking has stopped. This hypothesis is novel and carries with it the implication of a heretofore unrecognized autoimmune component of smoking-associated diseases manifested through production of anti-idiotypic antibodies with tobacco-like activity.

Materials and Methods

Preparation and Partial Characterization of CSC

Cigarette smoke condensate was obtained from the Phillip Morris Company, Richmond, Virginia. Twenty-five g of condensate were extracted with 400 ml of phosphate-buffered saline (PBS) for 18 hours at 50 to 56°C. During the early hours of extraction, pH 7.4 was maintained by drop-wise addition of 0.1 N NaOH. After this period the pH remained stable. The extract was clarified by centrifugation and the supernate was extracted v/v with petroleum ether (Fisher Scientific, Pittsburgh, PA). The aqueous phase of this extraction was further extracted v/v with ethyl ether (Fisher Scientific). The aqueous phase was retained and stirred in a beaker in a fume hood to remove ether vapors. The extract was then concentrated with pressure on an Amicon PM10 membrane (Amicon Corporation, Danvers, MA). Solute that did not pass the PM10 filter and that which did were retained separately. The latter was then concentrated by pressure dialysis on an Amicon YM1 membrane. The fraction that did not pass this filter and the fraction that did were both retained separately. In this way, solutes that were >10,000 molecular weight (MW), between 10,000 MW and 1,000 MW, and <1,000 MW were obtained. The material used in these studies was from the <1,000 MW fraction. It is referred to as CSC. It was sized further on Sephadex G10 (Sigma Chemical Co., St. Louis, MO) and determined to be <700 MW. The concentration of CSC in mg/ml was determined by freeze drying and weighing. CSC contains little protein as measured with the Bradford assay⁸ but is very reactive in a tannin assay⁹ and most likely the majority of the reagent is polyphenolic. An equimolar complex of rutin, chlorogenic acid, and scopoletin, the three major polyphenol components of tobacco,¹⁰ has been identified in cigarette smoke and CSC may be related to this complex.¹¹ Before use, CSC was filtered through Detoxi-Gel (Pierce Biochemicals, Rockford, IL), to remove any endotoxin in the preparation. This step was critical because others have shown that lipopolysaccharide (LPS) in the presence of human serum as a source of LPS-binding protein will prime neutrophils.¹² As an additional precaution the studies reported here were performed in the absence of human serum. All of the media and buffers were purchased as endotoxin-free reagents.

The amount of tobacco-associated phenolics that a smoker inhales has been shown to range from 101 to 185 $\mu\text{g}/\text{cigarette}$.^{13,14} Smokers who smoke two packs a day could easily inhale up to 7,200 μg of polyphenols per day. Because these substances are of low molecular weight (300 to 700 daltons) and would be expected to

cross the pulmonary epithelium easily, the concentration of these substances in pulmonary capillary blood could well be in the range of the concentration used in these experiments. Studies on the pharmacokinetics of the polyphenolics in tobacco smoke after inhalation have not been reported in the scientific literature.

Preparation of TAIA

Tobacco glycoprotein (TGP) was extracted from tobacco leaves as described.¹⁵⁻¹⁷ TGP is a complex of tobacco leaf proteins and tobacco-associated polyphenols. Antibodies to TGP were generated in rabbits.¹⁸ The IgG fraction of the rabbit anti-TGP was isolated by affinity chromatography on immobilized Staphylococcal Protein A (Pierce Biochemicals). It was further purified by affinity chromatography and eluted from columns of TGP coupled to aminoalkyl-Sepharose columns with EDAC (Bio-Rad, Hercules, NY). BALB/c mice were immunized with the IgG fraction of rabbit anti-TGP in complete Freund's adjuvant. The monoclonal anti-idiotypic antibody was prepared by fusing myeloma cell line P3x63Ag8.653 (ATCC CRL1580) with spleen cells from these BALB/c mice. Anti-idiotypic antibodies were identified by enzyme-linked immunosorbent assay using purified rabbit anti-TGP IgG, rabbit anti-rutin IgG (prepared as described previously¹⁸), rabbit anti-bovine serum albumin, and pooled rabbit IgG as target antigens. Confirmation of the anti-idiotypic specificity of the TAIA was established by demonstrating that monoclonal antibodies reactive with rabbit anti-TGP IgG or rabbit anti-rutin IgG but not rabbit anti-bovine serum albumin IgG or normal rabbit IgG, could be competitively inhibited from binding to these antigens in the presence of TGP, rutin, or chlorogenic acid, polyphenols found in tobacco leaves. The clone that produced the monoclonal antibody with the TAIA specificity was subcloned and an antibody of IgG₁ subclass was isolated by chromatography on Bakerbond ABx columns (J. P. Baker Chemical Co., Phillipsburg, NJ). It is referred to as TAIA. Another mouse monoclonal of IgG₁ subclass that reacts with an IgG myeloma protein and IgG from pooled mouse serum was also isolated on Bakerbond ABx columns. They are referred to as anti-id control and normal mouse IgG control, respectively. Before use, all antibody preparations were filtered through Detoxi-Gel (Pierce Biochemicals), to remove any endotoxin in the preparation. Protein concentrations were determined using the Lowry assay.¹⁹

Isolation of Neutrophils from Peripheral Blood

Peripheral blood was collected in sodium heparin (14.3 usp units/ml) from nonsmoking laboratory personnel. The neutrophils were isolated immediately after the blood draw. Blood (3.0 ml) was layered over 4.0 ml of Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) in a 12-ml polystyrene tube. The blood was allowed to settle at room temperature for 1 hour at which time the upper plasma/cell layer was removed. The plasma/cell mixture was then layered over 3.0 ml of Ficoll-Paque in a 12-ml tube and

centrifuged at $1,000 \times g$ for 15 minutes at 18°C. The neutrophils at the bottom of the tube were washed three times at 4°C in Hanks' balanced salt solution (HBSS) (Life Technologies, Inc., Grand Island, NY) at 4°C. Residual red cells were lysed with hypotonic saline, neutrophils were washed again in HBSS and resuspended in the appropriate buffer. All experiments were completed within 3 hours of neutrophil isolation.

Flow Cytometric Analysis of CD11b/18

Anti-CD11b labeled with phycoerythrin, a marker for expression of CD11b/18, was purchased from Becton Dickinson (Mountain View, CA). Neutrophils were suspended to 5×10^6 cells/ml in PBS containing 5.0% glucose. A 0.2-ml aliquot of cells was mixed with 9 $\mu\text{g/ml}$ of CSC or 10 $\mu\text{g/ml}$ of TAIA or 10 $\mu\text{g/ml}$ normal mouse IgG or PBS and incubated for 10 minutes at 37°C in a shaking water bath. Samples were cooled and received 20 μl of anti-serum and 0.4 ml of ice-cold PBS and kept on ice for 10 minutes. To measure the effect of CSC on expression of CD11b/18 in whole blood, 9 $\mu\text{g/ml}$ CSC or PBS were mixed with 0.2 ml whole heparinized blood and incubated for 10 minutes at 37°C. Each sample received 20 μl of antiserum and was placed on ice for 10 minutes. A Coulter Q Prep system (Coulter Corporation, Miami Lakes, FL) was used for lysing and fixing the stained cells. Analysis was performed using a Coulter EPICS Profile II. Results were expressed as a fold increase in CD11b/18 expression defined as the ratio of mean fluorescence intensity of cells incubated with CSC or TAIA and the mean fluorescence intensity of control cells incubated with PBS or control antibody.

Quantitation of fMLP Receptors

fMLP receptors on neutrophils were quantitated using an established assay.²⁰ [^3H]fMLP was purchased from New England Nuclear Life Sciences (Cambridge, MA). Aliquots (0.1 ml) of neutrophils at 1×10^6 cells/ml in HBSS, 1% heat-inactivated human AB+ serum and 5 mmol/L glucose were added to wells of microtiter plates. The cells were incubated with CSC at a final concentration of 9 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ TAIA, 10 $\mu\text{g/ml}$ anti-id control, or buffer at 37°C. Varying volumes of diluted [^3H]fMLP were added for final concentrations of 10 to 300 nmol/L. After 1 hour at 37°C the cells were collected on Whatman GF/B glass fiber filters using a PHD Cell Harvester (Cambridge Technology, Watertown, MA). The filters were counted using a Packard 2000CA Liquid Scintillation Counter (Meriden, CN). Scatchard plot analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA) and the number of fMLP receptors per cell was calculated.

Quantitation of Albumin Released in Response to CSC

Neutrophils isolated from peripheral blood were washed five times in PBS and suspended to 5×10^6 cells/ml in

PBS. Cells were incubated at 37°C in a shaking water bath with either CSC (9 $\mu\text{g/ml}$) or PBS for 0, 5, and 10 minutes. The cells were centrifuged, the supernate was collected and analyzed for human albumin in a sandwich enzyme-linked immunosorbent assay. Ninety-six well enzyme-linked immunosorbent assay plates were coated with rabbit anti-human albumin (DAKO, Carpinteria, CA) at a final concentration of 5 $\mu\text{g/well}$. SuperBlock (Pierce Biochemicals) diluted in PBS was used as the blocking buffer. A standard curve was included in each assay using human albumin from ICN HSA Biochemicals (Costa Mesa, CA). The assay was developed using rabbit anti-human albumin horseradish-peroxidase-conjugated antibody (DAKO) and O-phenylenediamine (Sigma Chemical Co.) as the substrate. The standard curve was generated using StatView (Abacus Concepts, Inc., Berkeley, CA)

Quantitation of Neutrophil Superoxide Production

An assay to quantitate superoxide produced by neutrophils after stimulation with fMLP was performed as described.^{21,22} Purified neutrophils at 6×10^5 cells/ml in HBSS were incubated for 10 minutes at 37°C with either 9 $\mu\text{g/ml}$ of CSC, 10 $\mu\text{g/ml}$ of TAIA, or 10 $\mu\text{g/ml}$ antibody control or PBS. Superoxide dismutase (Sigma Chemical Co.) at a final concentration of 750 U/ml or buffer and cytochrome c were added. Cells were stimulated with 1 $\mu\text{mol/L}$ fMLP in a final volume of 1 ml for 1 hour at 37°C. After centrifugation, the supernatant was collected and read on a UV/VIS Spectrometer Lambda 10 (Perkin-Elmer, Norwalk, CT). The amount of superoxide anion produced per neutrophil was calculated from the amount of cytochrome c reduction inhibited by superoxide dismutase.

Quantitation of Neutrophil Elastase

Aliquots of 5×10^5 cells received CSC at a final concentration of 9 $\mu\text{g/ml}$, TAIA at 10 $\mu\text{g/ml}$, antibody control at 10 $\mu\text{g/ml}$ or PBS. After incubation for 10 minutes at 37°C, fMLP was added to each tube at a final concentration of 1 $\mu\text{mol/L}$ and incubation continued for 45 minutes with shaking. The tubes were centrifuged and the elastase activity in the cell supernate was measured. L-Pyroglutamate-S-prolyl-L-valine-p-nitroaniline (S2484; Chromogenix, Molndal, Sweden), a synthetic substrate that is specific at pH 8.3 for neutrophil elastase was used in these studies. The assay for measuring elastase using the synthetic substrate was performed at 37°C according to published procedures.²³ In preliminary experiments performed to determine whether CSC interfered with the synthetic substrate assay for elastase, neutrophil elastase was prepared as described²⁴ and allowed to cleave S2484 in the presence or absence of CSC. CSC was found to not interfere with the enzymatic activity of elastase as measured in this assay. International units/ml (IU/ml) of elastase were calculated using $\Delta\text{O.D./minutes}$ and an absorption coefficient for p-nitroaniline of 9.9 $\text{cm}^2/\text{mmol/L}$ at 405 nm.

Figure 1. Expression of CD11b/18 on neutrophils was measured after priming with CSC and TAIA. The control for neutrophils primed with CSC was PBS and the control for TAIA was an antibody of matched isotype (Con). Samples were analyzed by flow cytometry and the fold increase in expression of CD11b/18 was calculated by dividing the mean channel of fluorescence of the primed cells by the mean channel of fluorescence of the appropriate control. Results represent a mean $\pm$ SEM of at least nine separate experiments. Expression of CD11b/18 is significantly ($P < 0.01$) increased on the primed cells.

Figure 2. Expression of fMLP receptors on purified neutrophils after priming by CSC and TAIA was significant ($P < 0.05$) compared with the controls. Control cells were treated with PBS or the anti-id control (Con). The results represent the mean $\pm$ SEM of five separate experiments.

Statistical Analysis

The number of times each experiment was performed is included with the results for the experiment. Statistical differences (*t*-test), means, and SEM were calculated using StatView (Abacus Concepts, Inc.). In these studies, the threshold *P* value for statistical significance is $P < 0.05$.

Results

Effect of CSC and TAIA on Expression of CD11b/18 and fMLP Receptors on Neutrophils

Neutrophils were mixed with CSC, TAIA, anti-id control, or PBS and incubated for 10 minutes at 37°C in a shaking water bath, cooled on ice, stained with phycoerythrin-labeled anti-CD11b, and analyzed by flow cytometry. The results are shown in Figure 1. After incubation with CSC or TAIA, CD11b/18 expression on the neutrophils more than doubled ($P < 0.01$). To rule out the effect of CSC on expression of CD11b/18 on neutrophils being an artifact associated with the use of purified cells, CSC was added to whole blood collected in heparin. Also shown in Figure 1, after 10 minutes at 37°C there was a threefold increase ($P < 0.01$) in expression of CD11b/18 on neutrophils in whole blood. fMLP receptor expression was also measured on purified neutrophils primed with CSC and TAIA. As shown in Figure 2, after incubation with CSC or TAIA, the number of fMLP receptors on the neutrophils doubled compared to the controls ($P < 0.05$).

Release of Albumin from Neutrophils during Priming

CD11b/18, fMLP receptors, alkaline phosphatase, cytochrome b_{558} , albumin, and other plasma proteins are found in secretory vesicles, the most readily mobilized compartment in neutrophils.²⁵ Our data suggest that one effect of CSC and TAIA on neutrophils is to mobilize these vesicles to the plasma membrane resulting in translocation of CD11b/18 and fMLP receptors to the cell surface. As part of this process, the albumin contained within the secretory vesicles is released into the cell supernate.²⁵ Neutrophils isolated from peripheral blood were washed extensively to remove serum proteins bound to the cell membrane. Baseline albumin concentrations were achieved by adding PBS or CSC to neutrophils at 4°C, followed by immediate centrifugation. As shown in Figure 3, there is some release of albumin from cells incubated with PBS. This is not unexpected because a slight increase in expression of CD11b/18 occurs normally when purified cells are incubated at 37°C.²⁶ In the presence of CSC, however, after 5 minutes there was a 1.6-fold increase in albumin in the supernate compared with cells incubated with PBS. By 10 minutes, 2.5-fold more albumin had been released from the CSC-treated cells. These results provide evidence that one effect of CSC and TAIA is to mobilize secretory vesicles thereby increasing CD11b/18 and fMLP receptor expression on the primed cells.

Figure 3. Release of albumin from purified neutrophils during priming by CSC. Results represent the mean $\pm$ SEM of nine separate experiments. Significantly ($P < 0.001$) more albumin was released from the neutrophils after 5 and 10 minutes incubation with CSC (▲) compared with cells incubated in PBS (●).

Production of Superoxide by Neutrophils Primed with CSC and TAIA

Production of superoxide by neutrophils primed with CSC or TAIA before stimulation with fMLP was quantitated using a superoxide dismutase-inhibitable cytochrome c assay. As shown in Table 1, the neutrophils primed with CSC produced 50% more superoxide anion per cell which was a significant increase ($P < 0.003$) over the response of unprimed cells. Cells primed with TAIA produced 20% more superoxide than cells preincubated with a control IgG ($P < 0.003$).

Table 1. Production of Superoxide Anion in Response to fMLP by Neutrophils Primed with CSC and TAIA

Prime	n	Superoxide anion $\times 10^{10}/\text{PMN}$	SEM	Significance
PBS	10	1.01	0.05	
CSC	17	1.52	0.11	$P < 0.003$
nmIgG	6	1.31	0.07	
TAIA	8	1.57	0.07	$P < 0.003$

Table 2. Release of Elastase by Neutrophils Primed with CSC and TAIA

Prime	Stimulate	n	Elastase IU/ml $\pm$ SEM	Fold increase over unprimed control	Significance
PBS	PBS	5	2.67 $\pm$ 0.372		
CSC	PBS	5	3.19 $\pm$ 0.319		n.s.
PBS	fMLP	17	3.82 $\pm$ 0.235		
CSC	fMLP	17	6.20 $\pm$ 0.498	1.94	$P < .001$
nmIgG	PBS	4	5.14 $\pm$ 0.393		
TAIA	PBS	4	5.38 $\pm$ 0.416		n.s.
nmIgG	fMLP	17	6.10 $\pm$ 0.357		
TAIA	fMLP	17	11.24 $\pm$ 0.794	2.09	$P < .001$

The Effect of CSC and TAIA on Release of Neutrophil Elastase in Response to fMLP

Neutrophil elastase has been implicated as a major contributor to the inflammatory process especially in inflammatory lung diseases like COPD where it functions to destroy the architecture of the alveolar walls. Neutrophils were primed with CSC or TAIA and release of elastase after addition of fMLP was quantitated. As shown in Table 2, there was a 1.94-fold increase over control ($P < 0.001$) in elastase release when neutrophils were primed with CSC. Neutrophils primed with TAIA showed a 2.09-fold increase ($P < 0.001$) in elastase release. In these experiments, neutrophils were incubated with the priming agent for 10 minutes followed by 45 minutes of incubation with fMLP or PBS. There was some elastase released from the cells in the absence of fMLP but the difference between spontaneous release of elastase in the presence of CSC or PBS was not significant (Table 2). This can be interpreted to mean that CSC alone is not functioning as an activating agent. Similarly, even though spontaneous release of elastase in the presence of mouse IgG control or TAIA was increased compared with CSC or PBS the response to the two mouse immunoglobulin reagents was not significantly different.

Effect of SB203580, a Specific Inhibitor of MAPK p38A Activation and Phosphorylation, on Neutrophil Priming by CSC

A role for MAPK p38 activation and phosphorylation in neutrophil priming by CSC and TAIA was examined using SB203580 (Calbiochem), a specific p38 inhibitor.^{27,28} Neutrophils at 10^7 cells/ml were mixed with buffer or SB203580 at a final concentration of 500 nmol/L and incubated for 10 minutes at 37°C. Aliquots (0.2 ml) of inhibitor-treated or -untreated cells received CSC at 9 $\mu\text{g}/\text{ml}$ or PBS. After 10 minutes at 37°C, the cells were cooled on ice, stained with phycoerythrin-labeled anti-CD11b, and analyzed by flow cytometry. As shown in Figure 4, CSC caused a twofold or 100% increase in CD11b/18 expression ($P < 0.01$). With the cells that had been pretreated with SB203580, the increase in expression of CD11b/18 after incubation with CSC was only 20% and did not differ significantly from the PBS control.

In the second set of experiments, neutrophils were primed in the presence of SB203580 and superoxide

Figure 4. Expression of CD11b/18 on neutrophils treated with SB203580 before priming with CSC. This is representative of four experiments, performed in triplicate, with similar results.

production after stimulation with fMLP was measured. Results are summarized in Table 3 and show a significant ($P < 0.03$) increase in superoxide production, as would be expected, when neutrophils were primed with CSC. When the cells were pretreated with SB203580 before CSC their response to fMLP was similar to the response of unprimed cells suggesting SB203580 was preventing the priming process from occurring. The observation that SB203580 treatment alone did not significantly reduce the ability of the neutrophils to produce superoxide in response to fMLP further supports its role as an agent that interferes with the priming mechanism. The results suggest that blocking activation and phosphorylation of MAPK p38 with a specific inhibitor prevents neutrophil priming by CSC. In these experiments, the effect of SB203580 was to abrogate priming by CSC and it did not significantly affect the ability of the cells to respond to fMLP activation as measured by superoxide production.

Discussion

The work presented here is a continuation of studies throughout the past several years examining the effect of

agents in tobacco on the inflammatory system. The long range goal of these studies has been to define specific points in the sequence of events that occur during inflammation that may be modified because of cigarette smoking. We have reported previously that TGP, a protein-polyphenol complex isolated from flue-cured tobacco leaves and found in smoke activates Factor XII (Hageman factor)-dependent pathways of coagulation, fibrinolysis, and generation of bradykinin.¹⁷ We also reported that TGP activates the classical complement pathway in the absence of specific antibody by binding directly to the globular region of C1q.¹⁶ We now demonstrate that studies showing neutrophil priming by exposure to whole cigarette smoke *in vivo*¹⁻³ can be duplicated *in vitro* using purified neutrophils and a low molecular weight polyphenol-rich material, CSC, extracted from CSC. Further, the priming effect of CSC on neutrophils can be mimicked by a TAIA. Cells primed with CSC and TAIA showed a two-fold increase in release of elastase in response to fMLP. Further, there was a 20 to 50% increase in superoxide production coupled with a twofold increase in fMLP receptors. These results are similar in magnitude to the responsiveness of neutrophils primed *in vivo* and isolated from the peripheral blood of cigarette smokers; neutrophils isolated from smokers generated 13% more superoxide in response to fMLP than nonsmoking controls and a 30% increase in expression of fMLP receptors.¹ In Figure 5, the concept of tobacco anti-idiotypic antibodies mimicking CSC to prime neutrophils is diagrammatically illustrated.

One effect of CSC and TAIA is to mobilize neutrophil secretory vesicles, thereby increasing CD11b/18 and fMLP receptor expression on the primed cells. The mechanism of the contribution of fMLP receptors on cigarette smoke-primed neutrophils, *in vivo* and *in vitro*, to the increase in responsiveness of these cells to fMLP activation is unclear. While it is tempting to propose a cause-and-effect mechanism, studies with other priming agents, including ATP, platelet activating factor, tumor necrosis factor- α , interleukin (IL)-8, LPS, and granulocyte macrophage-colony stimulating factor,²⁹ have demonstrated that an increase in fMLP receptors is not necessarily required for an increased oxidative burst response to fMLP activation. Zimmerli et al³⁰ using neutrophils primed with LPS found neutrophil oxidative burst was increased in response to C5a in the absence of up-regulation of C5a receptors. These results argue against there being a direct association between an increase in receptor number and the responsiveness of primed cells. Studies on the response of CSC- and TAIA-primed cells to agents other than fMLP with receptors that are not so easily mobilized are currently underway.

Up-regulation and activation of CD11b/18 on cigarette smoke-primed neutrophils may be very important for recruiting and maintaining these cells at inflammatory foci. A result of up-regulation and activation of CD11b/18 coupled with an increase in ICAM-1 in response to infection or injury would be increased leukocyte adhesion, emigration, and tissue damage in smokers. We used the increase in expression of CD11b/18 in response to CSC as a marker of priming and showed that it could be pre-

Table 3. Superoxide Production in Response to fMLP by Neutrophils Primed in the Presence and Absence of SB203580

Prime	SB203580	n	Superoxide anion $\times 10^{10}/\text{PMN}$	SEM	Significance*
PBS	No	4	0.89	0.13	
PBS	Yes	4	0.75	0.12	$P = 0.23$
CSC	No	4	1.17	0.19	
CSC	Yes	4	0.82	0.13	$P < 0.02$

*Neutrophils primed with CSC produced significantly more superoxide when stimulated with fMLP (PVS versus CSC, $P < 0.03$). The response of cells primed in the presence of SB203580 to fMLP was not significantly different from the response of unprimed cells (CSC-SB203580 versus PBS, $P = 0.22$).

Figure 5. a: Purified rabbit antibodies reactive with polyphenols in tobacco were used as the antigen for generation of mouse monoclonal anti-idiotypic antibodies. An anti-idiotypic antibody (TAIA) was generated with functional activity similar to CSC, a complex of tobacco polyphenols. **b:** Both CSC and TAIA prime neutrophils to cause increased expression of CD11b/18 and fMLP receptors. Neutrophils primed with either agent produce significantly more superoxide and released significantly more elastase than unprimed cells when stimulated with fMLP.

vented using SB203580, an inhibitor specific for MAPK p38 activation and phosphorylation. Further, enhancement of the oxidative burst of neutrophils primed with CSC and challenged with fMLP was abrogated in the presence of SB203580. It is not surprising that CSC, a plant-derived product would use MAPK pathways. Other polyphenols extracted from green tea have been shown to activate ERK and JNK MAPK in human hepatoma HepG2 cells.³¹ In plants, MAP kinases represent an ancient signaling mechanism, are implicated in a wide variety of biological responses, and play a role in signaling of abiotic stress, pathogens, and plant hormones.³²

The premise for our work is that in smokers, primed neutrophils contribute to the occurrence and progression of cigarette smoking-associated diseases that are inflammatory in origin. After cessation of smoking, the presence of tobacco anti-idiotypic antibodies functionally similar to priming agents in cigarette smoke may continue to drive the process. A variety of anti-idiotypic antibodies have

been described in human serum. For example, pooled normal human IgG prepared for therapeutic use has been shown to contain antibodies reactive with anti-mitochondrial antibodies from patients with Graves disease, postpartum thyroiditis, and autoimmune thyroiditis through idiotype-anti-idiotype interactions.³³ As part of the immune network, the natural occurrence of anti-idiotypic antibodies may be extremely common. Generation of tobacco anti-idiotypic antibodies would require an initial immune response to tobacco antigens. When challenged intradermally with a TGP preparation, at least one third of smokers and nonsmokers tested developed an immediate, cutaneous hypersensitivity wheal and flare reaction, indicating an IgE-mediated response.³⁴ This suggests that an immune response to tobacco is not a rare event. Constituents of cigarette smoke may help drive the immune system by stimulating release of IL-1 α , IL-1 β , IL-6, and PDGF from macrophages.³⁵ They also cause release of IL-4 from TH₂ lymphocytes which drives

B-cell immunoglobulin production.^{36,37} As a general rule, removing the antigen should result in eventual clearance of antibodies to tobacco with consequent reduction in production of anti-idiotypic antibodies. Even after smoking has stopped, this network balancing may not take place because there will be continued stimulation of the immune system by smaller amounts of antigen in side-stream smoke³⁸ coupled with cross-reactive antigens from other environmental sources.¹⁸

Other examples of anti-idiotypic antibodies functioning like the original antigen include antibodies with insulin-like activity,³⁹ antibodies that mimic tumor antigens, and an anti-idiotypic antibody that mimics the functional activity of Paclitaxel (Taxol) a low molecular weight anti-tumor agent.⁴⁰ The Paclitaxel anti-idiotypic antibody model is an example especially relevant to our work because both Paclitaxel and tobacco polyphenols share aromatic ring structure and bear no resemblance to immunoglobulin molecules. Yet both the Paclitaxel-like anti-idiotypic antibody and TAIA display functional activity identical to the original antigen.

In the next phase of our studies we will quantitate tobacco anti-idiotypic antibodies in serum from healthy smokers and smokers with smoking-associated disorders like COPD. In preliminary studies, six of 11 (55%) patients with clinically documented COPD (all previous or current smokers) and one of nine (11%) serum samples from disease free, long-term smokers (up to 45 years smoking history) had TAIA as measured in an enzyme-linked immunosorbent assay. This study will be expanded to include more patients, TAIA will be isolated by affinity chromatography and the functional activity of the TAIA as a neutrophil-priming agent will be measured. The hypothesis is that some smokers produce TAIA functionally similar to CSC that drive progression and even aid in initiation of cigarette smoking-associated inflammatory diseases long after smoking has stopped. This hypothesis is novel but the *in vitro* results provide evidence that the postulate it is based on is sound. It is now critical to demonstrate antibodies to tobacco antigens and anti-idiotypic antibodies in patients and correlate these findings with course and severity of smoking-associated diseases.

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