The Involvement of Peripheral Polymorphonuclear Leukocytes in the Oxidative Stress and Inflammation among Cigarette Smokers

Shifra Sela PhD, Revital Shurtz-Swirski PhD, Jamal Awad MD, Galina Shapiro MSc, Lubna Nasser MSc, Shaul M. Shasha MD and Batya Kristal MD

Eliachar Research Laboratory, Nephrology and Hypertension Unit, Internal Department VI, Western Galilee Hospital, Nahariya, Israel
Affiliated to Technion Faculty of Medicine, Haifa, Israel

Key words: inflammation, necrosis, oxidative stress, polymorphonuclear leukocytes, smoking load

Abstract

Background: Cigarette smoking is a well-known risk factor for the development of endothelial dysfunction and the progression of atherosclerosis. Oxidative stress and inflammation have recently been implicated in endothelial dysfunction.

Objectives: To assess the concomitant contribution of polymorphonuclear leukocytes to systemic oxidative stress and inflammation in cigarette smokers.

Methods: The study group comprised 41 chronic cigarette-smoking, otherwise healthy males aged 45.0 ± 11.5 (range 31–67 years) and 41 male non-smokers aged 42.6 ± 11.3 (range 31–65) who served as the control group. The potential generation of oxidative stress was assessed by measuring the rate of superoxide release from separated, phorbol 12-myristate 13-acetate-stimulated PMNL and by plasma levels of reduced (GSH) and oxidized (GSSG) glutathione. Inflammation was estimated indirectly by: a) determining the *in vitro* survival of PMNL, reflecting cell necrosis; b) *in vivo* peripheral PMNL counts, reflecting cell recruitment; and c) plasma alkaline phosphatase levels, indicating PMNL activation and degranulation.

Results: PMA-stimulated PMNL from cigarette smokers released superoxide at a faster rate than PMNL from the controls. Smokers had decreased plasma GSH and elevated GSSG levels. *In vitro* incubation of control and smokers' PMNL in sera of smokers caused necrosis, while control sera improved smoker PMNL survival. Smokers' PMNL counts, although in the normal range, were significantly higher than those of controls. Plasma ALP levels in smokers were significantly higher than in controls and correlated positively with superoxide release and PMNL counts.

Conclusions: Our study shows that PMNL in smokers are primed *in vivo,* contributing concomitantly to systemic oxidative stress and inflammation that predispose smokers to endothelial dysfunction, and explains in part the accelerated atherosclerosis found in smokers.

IMAJ 2002;4:1015–1019

For Editorial see page 1077

Cigarette smoking is a well-known risk factor for the development and progression of atherosclerosis [1]. The mechanism that predisposes cigarette smokers to organ injury and atherosclerosis is multifactorial and mostly unknown. Endothelial dysfunction appears to have a central role in the pathogenesis of atherosclerosis and is frequently described in smokers [2]. Oxidative stress and inflammation are among the mechanisms underlying endothelial dysfunction in clinical states associated with atherosclerosis [3]. Evidence for increased systemic oxidative stress in smokers is based mainly on decreased concentrations of endogenous antioxidants, increased oxidants and increased level of oxidation adducts [4], while evidence for systemic inflammation relies on increased levels of fibrinogen and C-reactive protein [5].

The peripheral polymorphonuclear leukocyte-mediated injury in smokers caused by inflammation and oxidative stress is known, but the majority of the reports focus on local airway pathologies [6], while only a few relate to the role of these cells in systemic oxidative stress [7]. Cigarette smoke constituents were also found to impair PMNL bactericidal activity [8]. Nicotine *in vitro* potentiated PMNL to release superoxide [8], while superoxide, itself derived from smoke, impaired the oxidative metabolism of PMNL [9].

We have previously established that peripheral PMNL, when primed, display enhanced superoxide release contributing to systemic oxidative stress. Simultaneously, these cells start a cascade of systemic inflammatory events, a process initiated by enhanced necrotic cell death, promoting chemotaxis and PMNL recruitment [10–12]. We have shown that primed PMNL are the common denominator in inducing systemic oxidative stress and chronic inflammation in clinical disorders known to be associated with endothelial dysfunction, accelerated atherosclerosis, and increased cardiovascular morbidity and mortality. Among these disorders are essential hypertension, diabetes mellitus type 2, chronic renal failure, and end-stage renal disease treated with hemodialysis [10–12].

Thus, the aim of the present study was to establish whether smoking could be included in the above-mentioned pathologies, predisposing smokers to endothelial dysfunction and atherosclerosis by the presence of concomitant systemic oxidative stress and chronic inflammation caused by their PMNL.

Patients and Methods

Two groups of subjects were enrolled in this study: 41 healthy male chronic cigarette smokers aged 45.0 ± 11.5 (range 31–67 years), and 41 male non-smokers aged 42.6 ± 11.3 (range 31–65) who served as the controls. The smoking load or the chronic cigarette consumption of each individual was determined by calculating the

PMNL = polymorphonuclear leukocytes
PMA = phorbol 12-myristate 13-acetate
GSH = reduced glutathione
GSSG = oxidized glutathione
ALP = alkaline phosphatase

number of cigarette-packs smoked per day multiplied by the number of smoking years (pack-year) [13]. Smokers included in this study had to smoke no less than one pack of cigarettes (20 cigarettes) per day, had a pack-year greater than 10, and were allowed to smoke until 30 minutes before their blood withdrawal to prevent acute smoke changes as described by Blann et al. [14]. All subjects underwent a clinical examination and routine laboratory blood tests to rule out the presence of any coexisting disease. All studied subjects had normal blood pressure (<130/80 mmHg), normal kidney and liver function, and normal plasma levels of glucose, cholesterol and triglycerides. Subjects with evidence of infection or systemic diseases were excluded; occult infection was ruled out by assaying sCD14 (Quantikine Kit, R&D, USA). All participants included in the study had sCD14 levels within the normal range. All subjects signed an informed consent prior to their participation in the study and their blood donation. Blood was withdrawn from all the subjects for leukocyte and differential counts, plasma and serum separation, and for PMNL isolation.

Blood withdrawal and PMNL and sera separation

PMNL isolation was performed from a 20 ml heparinized (50 U/ml) blood sample according to the method of Klebanoff and Clark [15] with modifications [10–12]. The separated PMNL (>98% pure, approximately 10^8 cells per isolation) were resuspended in a minimal volume (0.1–0.3 ml) of Hank's balanced salt solution, immediately counted, and diluted to a final volume of 1 ml with HBSS containing 0.5% glucose, according to different experimental needs. Sera from smokers and controls were frozen at 70°C and saved for *in vitro* survival assays (see below).

Oxidative stress

Determination of the rate of superoxide release from separated PMNL. The rate of superoxide release was assayed for 90 min: a) under basal conditions, b) after stimulation with 0.32×10^{-7} M phorbol 12-myristate 13-acetate (Sigma, USA), and c) after stimulation with zymosan. The assay is based on superoxide dismutase inhibitable reduction of 80 μM cytochrome C (Sigma) to its ferrous form [16] with slight modifications [10–12].

Determination of glutathione levels in plasma. Glutathione determinations were carried out in randomly chosen 18 age-matched subjects of each group (age: smokers 43.7 ± 13 years, controls 46.1 ± 7.3 years), and performed as previously described [10,12]. Briefly, two separate measurements were carried out: evaluation of a) the total glutathione level in plasma [(GSH)+2(GSSG)] and b) the oxidized form (GSSG). The plasma level of GSH was calculated as the difference between the total glutathione and the oxidized 2(GSSG).

Indirect measurements of inflammation

White blood cells and PMNL counts. WBC and PMNL counts of blood withdrawn in EDTA from both smokers and controls were performed by using a Coulter STKS counter (Miami, FA, USA).

PMNL survival in vitro as measure for necrosis. For these studies, PMNL were separated (10^7/ml) from 22 subjects randomly chosen from each group (age 47.07 ± 12.2 and 41.7 ± 11.6 years for smokers and controls respectively). Control PMNL were cross-incubated with pooled autologous control sera or heterologous smoker sera, and smoker PMNL were cross-incubated with pooled autologous smoker sera or heterologous control sera (25% v/v diluted with HBSS) for 90 minutes. PMNL were counted by Coulter counter before and after 90 min incubation. Cell viability was confirmed by trypan blue (0.1% w/v) exclusion. Survival is expressed as the ratio of cell counts before and after the 90 min incubation with sera (%).

Statistical analysis

Data are expressed as mean ± SEM. Differences between controls and smokers were tested by unpaired Student's *t*-test. Analysis of correlation between different study parameters was performed using linear and non-linear regression analysis. $P < 0.05$ was considered significant.

Results

Oxidative stress

No superoxide release from resting PMNL of either controls or smokers could be detected under basal conditions during 90 minutes. Rates of superoxide release from PMA- and zymosan-stimulated PMNL separated from controls and smokers are depicted in Figure 1. In smokers, the rate of superoxide release from PMA-stimulated PMNL was significantly faster than from control PMNL ($P = 0.0001$). To distinguish between this PMA-stimulated, "non-targeted" superoxide release and a bactericidal phagocytic activity, the rate of superoxide release from PMNL exposed to the specific stimulant, zymosan, was also followed. In contrast to PMA stimulation, a significant impairment in the response to zymosan was found in PMNL of smokers compared to those of controls ($P = 0.005$).

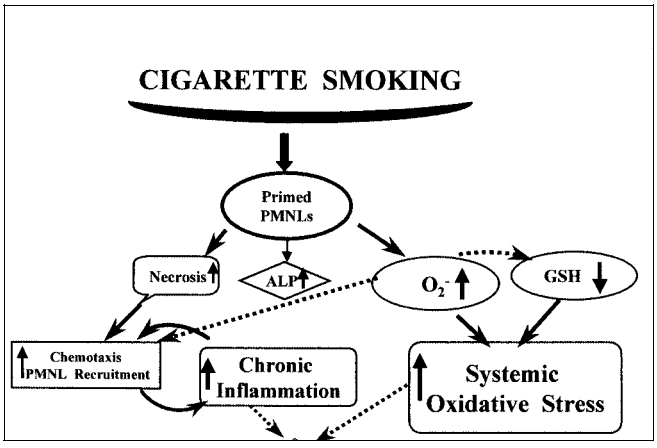

Figure 1. Rate of superoxide release by stimulated control and smokers' PMNL. Rate of superoxide was measured as described in Methods and expressed as nmoles/10 min/10^6 cells. Data are mean ± SEM. * $P = 0.0001$, PMA-stimulated control PMNL vs. smokers' PMNL; ** $P = 0.005$, zymosan-stimulated control PMNL vs. smokers' PMNL. NC = normal control, CS = cigarette smokers.

HBSS = Hank's balanced salt solution
WBC = white blood cells

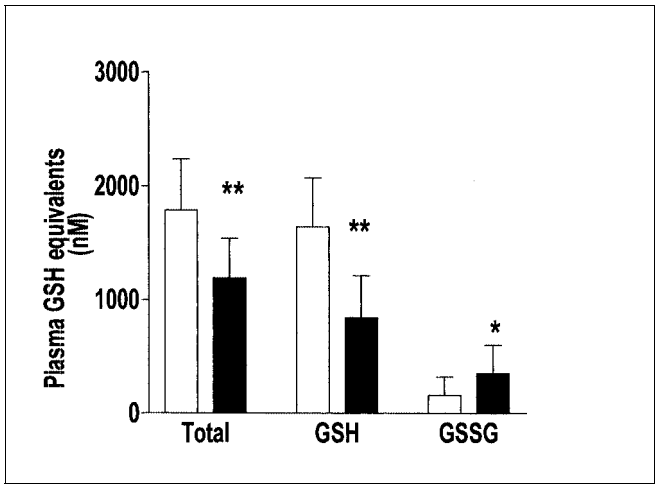

Figure 2. Plasma levels of total, reduced and oxidized glutathione from controls □ (n = 18) and smokers ■ (n = 18). Total, reduced and oxidized glutathione levels were measured as described in Methods. Data are mean ± SEM. * P < 0.001 vs. the control plasma, ** P < 0.0001 vs. the control plasma.

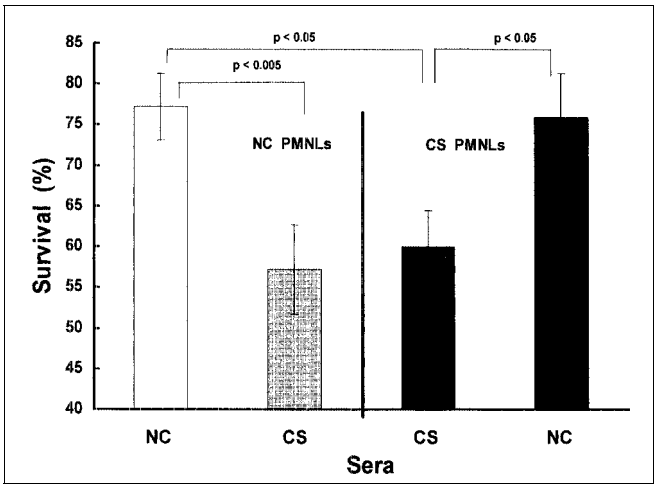

Figure 3. Percentage of survival of PMNL from controls (NC) and smokers (CS) after 90 minutes incubation in autologous and heterologous, normal or smokers' sera. Survival is expressed as the percentage of the ratio between the number of cells counted after 90 min incubation with sera and the cells counted at the beginning of incubation. Data are mean ± SEM.

Total and reduced plasma glutathione levels were significantly lower in smokers compared to controls (P < 0.0001) [Figure 2]. On the other hand, the level of oxidized glutathione increased significantly in smokers (P < 0.001) [Figure 2]. The presence of decreased GSH and increased GSSG in smokers is indicative of oxidative stress.

GSH and the rate of superoxide release from smokers were found to be negatively correlated (r = -0.42, P = 0.01), suggesting a causative relationship between these two parameters.

Indirect measurements of inflammation

WBC and PMNL counts were significantly higher in smokers than in controls (6.9 ± 0.2 vs. 7.7 ± 0.2 and 3.9 ± 0.1 vs. 4.4 ± 0.2, respectively; P < 0.05), although all values fell within the accepted normal range.

A significant reduction (P < 0.05) [Figure 3] in the survival of smokers' PMNL following incubation of 90 min in smokers' sera was evident, indicating cell necrosis, compared to 90 min incubation of control PMNL in control sera. Control sera significantly promoted (P < 0.05) the survival of smokers' PMNL to the level of control PMNL in autologous serum. Smokers' sera significantly caused necrosis or cell disintegration (P < 0.005) of control PMNL [Figure 3], expressed by decreased survival.

Correlation between oxidative stress and inflammation

A significant positive linear correlation between PMNL counts and the rate of superoxide release in smokers (r = 0.45, P < 0.003, n=41) was found, reflecting the substantial link between the two phenomena, namely concomitant contribution of the PMNL to oxidative stress and inflammation.

Relationships of ALP to oxidative stress and inflammation

Increased plasma levels of ALP, a PMNL-granule enzyme, in the absence of bone and liver diseases, reflect PMNL activation and degranulation [10]. Our smoking patients had normal liver function with no bone diseases; nevertheless they showed significantly higher plasma ALP, compared to NC (190.3 ± 5.2 vs.149 ± 11, respectively; P < 0.01), although all values fell within the accepted normal range. A significant positive linear correlation between smokers' plasma ALP levels and both PMNL counts (P < 0.0001, r = 0.79) and the rate of superoxide release (P = 0.012, r = 0.4) were found, reflecting PMNL activation and degranulation.

Relationships of smoking load (pack-year) to oxidative stress and inflammation

Using linear or non-linear regression analyses of smoking load and oxidative stress (either the rate of superoxide release or plasma GSH levels), we could show only an excessively large data spread of the two variables with no correlation between them. In addition, while drawing another correlation between inflammation (PMNL counts) and pack-year, no significant linear correlation was found.

Discussion

The present study illuminates the pivotal contribution of PMNL in smokers concomitantly to systemic oxidative stress and chronic inflammation, mechanisms that probably predispose smokers to endothelial dysfunction. While the existence of oxidative stress in smokers was previously demonstrated [4], our data suggest that PMNL is a new possible culprit in initiating a cascade of oxidative stress and inflammation in smokers. In this study PMA stimulation of PMNL in smokers reflects prior priming of these cells in the circulation, resulting in ROS generation and cell content release into the immediate milieu. These results are supported by the study of Kalra et al. [7] who reported an increased production of ROS in whole blood of smokers, partially contributed by primed PMNL, although a different methodology was used.

ROS = reactive oxygen species

Of interest is our finding that smokers have increased plasma ALP activity in the absence of liver and bone disease. This ALP activity correlates with both the number of PMNL and the rate of superoxide release, suggesting that activation and degranulation of PMNL may be the source for this PMNL-granule enzyme. A previous study from our laboratory showed similar findings for primed PMNL and increased PMNL-ALP activity in blood of essential hypertensive patients [10].

The ubiquitous antioxidant GSH is a useful plasma marker for an overall manifestation of a systemic oxidative injury [17]. We suggest that one of the underlying mechanisms whereby glutathione is oxidized in smokers is its consumption by ROS released from activated PMNL, since a significant linear correlation was found between the increased rate of superoxide release and the decrease in plasma GSH level. Our findings of reduced antioxidant capacity in smokers, as expressed by lower plasma GSH, is confirmed by the data found by Fuller et al. [18], demonstrating that antioxidant supplementation decreases the levels of oxidation products in smokers. The significant decrease in the total plasma GSH concentration in smokers compared to control subjects cannot be attributed to reduced hepatic synthesis or to increased renal excretion, as our subjects who smoke had no evidence of hepatic or renal malfunction. However, a lower amount of plasma total GSH can be explained by some irreversible oxidation of GSH to sulfur oxide adducts [19] that are not recognized and recovered by the GSH-reductase assay used in this study. To the best of our knowledge, only one study – that by Banerjee et al. [20] – has already reported reduced glutathione levels in the blood of smokers. However, plasma GSH and GSSG levels in smokers who have not yet developed clinical or laboratory signs of lung injury have not been reported to date.

Our observations emphasize that the oxidative metabolism of PMNL depends upon the nature or type of the stimulant. Using the same assay system, we have shown that the same PMNL preparation may respond in opposite directions to different stimuli: PMA stimulation of PMNL in smokers increases the rate of superoxide release, whereas in zymosan-stimulated PMNL the rate of release is slower compared to that in controls. As zymosan is used to assess the phagocytic function of PMNL, our findings also support the increased susceptibility to bronchopulmonary and upper respiratory tract infections in smokers [21].

Another important outcome of this study is the contribution of PMNL to chronic inflammation in smokers. Several reports imply that increased plasma levels of inflammation markers, such as plasma C-reactive protein and fibrinogen, correlate with cardiovascular morbidity [5]. In asymptomatic smokers, although an increase in C-reactive protein and fibrinogen was found, it was significant only in the group of smokers who also suffer from cardiovascular disease [5]. Our *in vivo* data of increased PMNL counts in smokers, combined with the decreased *in vitro* PMNL survival, implies that PMNL of smokers contribute to chronic inflammation already in an earlier stage. The leukoclastic effect of smokers' sera and the complete restoration of smokers' PMNL survival by control sera suggest that acquired, plasma-born smoke derivatives exist in the sera of smokers, responsible for the reduced survival of smokers' PMNL in sera of smokers. At the same time these factors may perpetuate the priming of PMNL, causing the enhanced release of superoxide. Some of these possible acquired factors such as nicotine [8] and saturated free fatty acids present in tobacco smoke were previously described [22]. The acquired leukoclastic activity of the smokers' sera starts a loop of inflammatory response: PMNL priming will cause their death by self-necrosis with an ongoing chemotaxis and cell recruitment. This recruitment is concomitant with increased oxidative stress, shown by a significant linear correlation between the increased rate of superoxide release and the increased PMNL count [10–12] [Figure 4]. The contribution of the PMNL to the chronic inflammatory state in smokers has never been considered, although an elevated but normal circulating leukocyte and PMNL count has already been reported in smokers. Thus, the present study offers a new explanation to this cell recruitment, manifested by a significant increase in smokers' peripheral PMNL count. Interestingly, the increase in leukocyte counts has been suggested as a predictor of myocardial infarction and lately became an index of the inflammatory state even when the counts lie within the normal range [23].

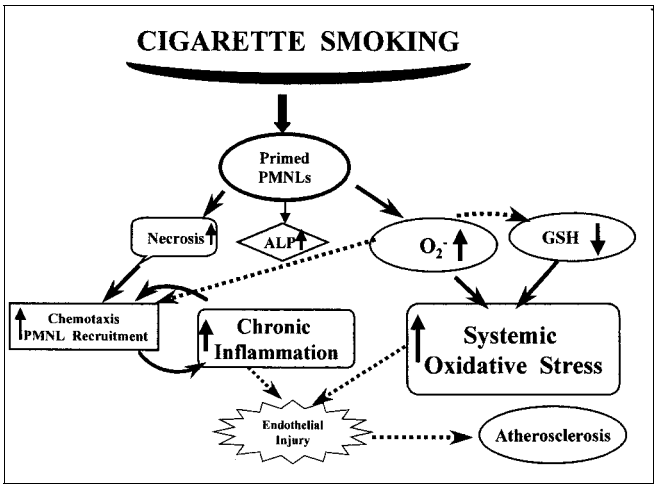

Figure 4. Illustrated summary of the concomitant contribution of PMNL to oxidative stress and inflammation in cigarette smokers.

This study shows that PMNL are activated or primed in smokers. Thus, if smoking is the major cause of PMNL activation, one would expect that the more an individual smokes, the effect of smoke on PMNL might be greater, as was found *in vitro* [8] and *in vivo* after acute smoking [14]. However, no correlation was found between the smoking load and either oxidative stress or inflammation among heavy smokers who smoke more than 20 cigarettes per day. This implies that the susceptibility of a smoking individual to injury caused by oxidative stress and inflammation is affected by additional factors – genetic, acquired, or demographic – whose nature has yet to be elucidated. Supportive of this idea is the report by Glantz and Parmley [24] showing that smoke-induced oxidative stress is probably more pronounced in subjects whose cardiovascular system lacks adaptation to smoke. Contradictory results were reported by Hulea et al. [25], who found that a low smoking load does not induce oxidative stress, whereas a heavy smoking load

does. Age differences between these studies can resolve this discrepancy.

In conclusion, primed PMNL are the common denominator in clinical states known to be associated with endothelial dysfunction and accelerated atherosclerosis. In patients with essential hypertension, diabetes mellitus type 2, chronic renal failure, and end-stage renal disease treated with hemodialysis [10–12], PMNL contribute to systemic oxidative stress and chronic inflammation – mechanisms known to induce endothelial dysfunction. This study adds cigarette smokers to the above-mentioned clinical disorders by focusing on the PMNL as the inducer of oxidative stress and inflammation, rendering an explanation as to why smoking is a risk factor for atherosclerosis.

Acknowledgement. The authors are grateful to Dr. J. Manaster and Dr. N. Goldstein for their critical review of the manuscript, to Mr. A. Ganem for recruiting cigarette smokers, and to Mrs. T. Kuritsky for proofreading and editing the manuscript. In addition, we wish to express our gratitude to Dr. J. Chezar for the hematologic tests and to Dr. T. Snitkovsky for the biochemical tests.

References

1. Howard G, Wagenknecht LE, Burke GL, et al. Cigarette smoking and progression of atherosclerosis: the Atherosclerosis Risk in Communities (ARIC) Study. *JAMA* 1998;279:119–24.
2. Hutchison S. Smoking as a risk factor for endothelial dysfunction. *Can J Cardiol* 1998;14(Suppl D):20–2D.
3. Alexander RW. Hypertension and the pathogenesis of atherosclerosis. Oxidative stress and the mediation of arterial inflammatory response: a new perspective. *Hypertension* 1995;25:155–61.
4. Abou-Seif MA. Blood antioxidant status and urine sulfate and thiocyanate levels in smokers. *J Biochem Toxicol* 1996;11:133–8.
5. Tracy RP, Psaty BM, Macy E, et al. Lifetime smoking exposure affects the association of C-reactive protein with cardiovascular disease risk factors and subclinical disease in healthy elderly subjects. *Arterioscler Thromb Vasc Biol* 1997;17:2167–76.
6. Nowak D, Ruta U, Piasecka G. Nicotine increases human polymorphonuclear leukocytes chemotactic response – a possible additional mechanism of lung injury in cigarette smokers. *Exp Pathol* 1990;39:37–43.
7. Kalra J, Chaudhary AK, Prasad K. Increased production of oxygen free radicals in cigarette smokers. *Int J Exp Pathol* 1991;72:1–7.
8. Jay M, Kojima S, Gillespie MN. Nicotine potentiates superoxide anion generation by human neutrophils. *Toxicol Appl Pharmacol* 1986;86:484–7.
9. Tsuchiya M, Thompson DF, Suzuki YJ, Cross CE, Packer L. Superoxide formed from cigarette smoke impairs polymorphonuclear leukocyte active oxygen generation activity. *Arch Biochem Biophys* 1992;299:30–7.
10. Kristal B, Shurtz-Swirski R, Chezar J, et al. Participation of peripheral polymorphonuclear leukocytes in oxidative stress and inflammation in patients with essential hypertension. *Am J Hypertens* 1998;11:921–8.
11. Kristal B, Shurtz-Swirski R, Shasha SM, et al. Interaction between erythropoietin and peripheral polymorphonuclear leukocytes in hemodialysis patients. *Nephron* 1999;81:406–13.
12. Shurtz-Swirski R, Sela S, Herskovits AT, et al. Involvement of polymorphonuclear leukocytes in oxidative stress and inflammation in type 2 diabetes. *Diabetes Care* 2001;24:104–10.
13. Bridges RB, Wyatt RJ, Rehm SR. Effects of smoking on inflammatory mediators and their relationship to pulmonary dysfunction. *Eur J Respir Dis Suppl* 1986;146:145–52.
14. Blann AD, Kirkpatrick U, Devine C, Naser S, McCollum CN. The influence of acute smoking on leucocytes, platelets and the endothelium. *Atherosclerosis* 1998;141:133–9.
15. Klebanoff SJ, Clark RA. Iodination by human polymorphonuclear leukocytes. *J Lab Clin Med* 1977;89:675–86.
16. Babior BM, Kipnes RS, Curnutte JJ. Biological defense mechanisms. The production by leukocyte of superoxide, a potential bactericidal agent. *J Clin Invest* 1973;52:741–4.
17. Ikegami KC, Lalonde Y, Youn K, Picard L, Demling R. Comparison of plasma reduced glutathione and oxidized glutathione with lung and liver tissue oxidant and antioxidant activity during acute inflammation. *Shock* 1994;1:307–12.
18. Fuller CJ, Grundy SM, Norkus EP, Jialal I. Effect of ascorbate supplementation on low-density lipoprotein oxidation in smokers. *Atherosclerosis* 1996;119:139–50.
19. Sies H. Glutathione and its role in cellular functions. *Free Radic Biol Med* 1999;27:916–21.
20. Banerjee KK, Marimuthu P, Sarkar A, Chaudhuri RN. Influence of cigarette smoking on vitamin C, glutathione and lipid peroxidation status. *Indian J Public Health* 1998;42:20–3.
21. Pizzichini E, Pizzichini MM, Efthimiadis A, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med* 1996;154:308–17.
22. Qian M, Eaton JW. Free fatty acids enhance hypochlorous acid production by activated neutrophils. *J Lab Clin Med* 1994;124:86–95.
23. Riyami BM, Tree SR, Kinsella J, et al. Changes in alveolar macrophage, monocyte and neutrophil cell profiles after smoke inhalation injury. *J Clin Pathol* 1990;43:43–5.
24. Glantz SA, Parmley WW. Passive smoking and heart disease. Mechanisms and risk. *JAMA* 1996;273:1047–53.
25. Hulea SA, Olinescu R, Nita S, Crocnan D, Kummerow FA. Cigarette smoking causes biochemical changes in blood that are suggestive of oxidative stress: a case-control study. *J Environ Pathol Toxycol Oncol* 1995;14:173–80.

Correspondence: Dr. B. Kristal, Head, Nephrology and Hypertension Unit, Western Galilee Hospital, Nahariya 22100, Israel.
Phone: (972-4) 992-0402
Fax: (972-4) 910-7482/69
email: krisdl@naharia.health.gov.il

Capsule

Genome sequence of the *Anopheles gambiae*

The enormous worldwide death rate from malaria has prompted an international consortium to sequence the genome of the principle vector, the mosquito *Anopheles gambiae*. Holt et al. present the results of the shotgun sequencing effort, which generated a sequence of 278 megabases representing 91% of the genome. These researchers present an initial functional annotation of the identified open reading frames and estimate that there are 14,000 protein-encoding transcripts for which they have multiple types of evidence. They also performed an expressed sequence tag analysis of genes whose expression changes after the mosquito feeds.

Science 2002;298:129