Atherosclerosis-related Responses to Cigarette Smoking in the Baboon

WALTER R. ROGERS, PH.D., ROBERT L. BASS III, PH.D., DONALD E. JOHNSON, PH.D., ARTHUR W. KRUSKI, PH.D., C. ALEX MCMANAHAN, PH.D., MILKA M. MONTIEL, M.D., GLEN E. MOTT, PH.D., ROBERT L. WILBUR, M.S., AND HENRY C. MCGILL, JR., M.D.

SUMMARY Thirty-six young adult male baboons (Papio cynocephalus) were fed an atherogenic diet (40% calories from lard, 1.5 mg cholesterol/kcal) and taught to puff by operant conditioning with water rewards. Eighteen baboons (smokers) were assigned randomly to smoke 43 cigarettes a day, and 18 baboons (shams) were assigned randomly to puff air under conditions equivalent to those of the experimental group. During months 14–19 of smoking, cigarette-smoking baboons had significantly higher carbon monoxide and thiocyanate concentrations in blood and cotinine concentrations in urine. There were no significant differences in serum total cholesterol, VLDL + LDL cholesterol, HDL cholesterol or triglyceride concentrations of smokers and shams. Smoking baboons had significantly higher fasting blood glucose concentrations and lymphocyte counts. Platelet count, platelet aggregation, food and water intake, and body weight were not significantly different in the two groups.

THE EPIDEMIOLOGIC ASSOCIATION of cigarette smoking with atherosclerosis and with the atherosclerotic diseases in the human is strong and consistent, but the mechanism of the cigarette smoking effect is unknown. Cigarette smoke contains many substances that may be involved, and cigarette smokers show many physiologic responses to smoking that could augment atherosclerosis.

We have undertaken an experiment using baboons, trained by operant conditioning techniques to smoke cigarettes in a human-like manner, to investigate whether cigarette smoking affects atherosclerosis and to examine several responses suspected of involvement in the pathogenesis of atherosclerosis. Dosimetry of cigarette smoke exposure was assessed by behavioral and chemical measures. Comparison of the smoking and control groups after 14 months of smoking disclosed several responses similar to those observed in humans that may be involved in atherogenesis.

Methods

Subjects

Thirty-six feral, young adult male baboons (Papio cynocephalus) were used. At the beginning of the experiment, the mean (± SD) weight of the baboons was 8.7 ± 1.34 kg, and the average age was estimated, based on dental eruption patterns, to be about 4 years (range 2–6 years; median 4 years). Pubertal baboons were chosen to simulate adolescent human smokers.

Experimental Design

A randomized block design with three blocks was used. The blocks were determined by arrival time (5-week intervals) of baboons from the importer. Animals within each block were assigned randomly to either experimental (cigarette-smoking) or control (sham-puffing) conditions. We treated both experimental and control groups alike, except that the control baboons puffed through cigarette filters that had a resistance to flow equivalent to that of a cigarette and the experimental baboons puffed on cigarettes. Both groups worked under the same puff duration and pressure requirements, magnitude of reward and schedule of reinforcements.

During weeks 1–5 we made baseline measurements of response variables, and in week 6 we placed all baboons on an atherogenic diet (40% calories from lard, 1.5 mg cholesterol/kcal). During weeks 15–30 the baboons learned how to earn water rewards by activating the Smoking Inhalation Response Indicator and Conditioner (SIRIC). In week 31, the smoking baboons began to receive cigarettes, and the control baboons received filters on the same schedule. Table I provides a summary of the response variables, the weeks after the beginning of smoking when the variables were measured, and the measurement methods used.

Experimental Cigarettes

P. Lorillard Company manufactured 638,000 cigarettes, designed to be representative of those marketed in the United States during 1974, especially for this experiment. The nonfilter cigarettes were 85 mm long, 25 mm in circumference, and marked with a 23-mm butt line. The cigarettes had an average weight of 118 g and a mean pressure drop of 76 mm of water.

The tobacco mixture included 45% flue-cured, 20% burley and 14% Turkish tobaccos plus 21%
Table 1. *Summary of Response Variables, Weeks Measurements Made, and Analytic Methods*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Week after start of smoking</th>
<th>Analytic method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking performance</td>
<td>60, 61, . . . 83, 84</td>
<td>SIRIC&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Puff volume, pressure and duration</td>
<td>70, 74, 78, and 82</td>
<td>PVAM (see text)</td>
</tr>
<tr>
<td>Carbon monoxide concentration in blood</td>
<td>61, 63, . . . 81, 83</td>
<td>Gas chromatographic&lt;sup&gt;20&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thiocyanate concentration in plasma</td>
<td>61, 65, . . . 77, 81</td>
<td>Colorimetric&lt;sup&gt;21&lt;/sup&gt;</td>
</tr>
<tr>
<td>Containing concentration and amount in 24-hour urine</td>
<td>61, 65, . . . 77, 81</td>
<td>Gas chromatographic&lt;sup&gt;22&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cadmium, lead, and zinc concentration in blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum cholesterol</td>
<td>60, 68, 76, and 84</td>
<td>Enzymatic&lt;sup&gt;26&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum triglyceride</td>
<td>60, 68, 76, and 84</td>
<td>Enzymatic&lt;sup&gt;27&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lipoprotein cholesterol</td>
<td>60 and 84</td>
<td>Precipitation&lt;sup&gt;28&lt;/sup&gt;</td>
</tr>
<tr>
<td>Erythrocyte and leukocyte count, hemoglobin concentration and hematocrit</td>
<td>60, 68, 76, and 84</td>
<td>Coulter S</td>
</tr>
<tr>
<td>Differential leukocyte count</td>
<td>60, 68, 76, and 84</td>
<td>Coulter Z</td>
</tr>
<tr>
<td>Platelet count</td>
<td>60, 68, 76, and 84</td>
<td>Coulter Z</td>
</tr>
<tr>
<td>Platelet aggregation</td>
<td>66 and 78</td>
<td>Optical density measurements of response to ADP and collagen&lt;sup&gt;29&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose concentration in blood, fasting</td>
<td>60 and 84</td>
<td>Enzymatic&lt;sup&gt;30&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviations: SIRIC = Smoke Inhalation Response Indicator and Conditioner; PVAM = Puff Volume Air Monitor.

reconstituted leaf. Glycerine and inverted sucrose were added at 2.8 and 5.3%, respectively, of the weight of tobacco and casings. The tobacco contained 2.2% nitrogen; 0.36% total volatile bases, 1.56% nicotine; 15.2% ash; and 9.7% of tobacco weight sugar (total reducing content of tobacco as glucose). Smoke from each cigarette, analyzed by Federal Trade Commission standardized methods,<sup>3</sup> contained, on the average, 26.7 mg tar, 1.54 mg nicotine, 31.6 mg total particulate matter, 3.32 mg H<sub>2</sub>O, 20.3 mg CO, 3.32 mg CO<sub>2</sub>, 163 μg NO, 258 μg HCN, and 118 μg acrolein.

**Cigarette Smoking and Instrumentation**

The SIRIC and the training procedures used with it have been described.<sup>4</sup> The animals are first taught to apply a negative pressure to a metal tube to obtain water. Once the negative pressure response is established, the animals are given access to the SIRIC, which senses negative pressure above a minimum level in the cigarette mouthpiece and measures the duration of negative pressure. If the puffing response exceeds the investigator-controlled pressure and duration minimum, a small water reward is dispensed to the animal through the water tube. The animals begin by making responses of 0.1 second at duration of 1.3 cm of water pressure, and then the minimum response criteria are gradually increased. During week 60 of this experiment, the baboons puffed with a mean minimum duration requirement of 3.4 ± 0.71 seconds) at 3.8 cm of water pressure. The baboons were given a cigarette or sham every 15 minutes for 12 hours each day. Because blood collection required use of an anesthetic, ketamine (Ketaset, Bristol Laboratories, Syracuse, New York), which disrupts puffing for several hours, the baboons averaged 43 cigarettes a day.

We measured puff volume, pressure and duration using a Puff Volume Air Monitor (PVAM). The principal components of the PVAM are a mouthpiece with a laminar flow element, two pressure transducers, signal conditioning cards and a microprocessor. Since the laminar flow element is of unique design, a single relatively large orifice (0.159 cm i.d.) rather than many relatively small holes, it provides a linear differential pressure proportional to flow without filtering of smoke constituents. As the baboon puffs, pressure drop across the laminar flow element is measured and that signal is integrated to give volume. Static pressure is measured at a separate tap, and the output of the static pressure transducer is used to determine puff duration. Mean static pressure is obtained by integrating the pressure signal and dividing by the measured puff duration. A puff-by-puff listing of the data, along with an indication if the puff met the SIRIC criterion, was recorded on tape cassette for later analysis. The device was calibrated by application of known pressures and flow rates before each use.
Statistical Techniques

We analyzed all responses by separate analyses. The multiple measurements of a response on a single baboon were regarded as a random sample from a multivariate normal distribution. Marginal distributions were examined and transformations made to approximate more closely the normality and homogeneity of variance. We tested the null hypothesis of no difference between means of smokers and shams, using a two-tailed alternative, with multivariate analysis of variance. Comparison of sham and cigarette smoking groups was based on observations made during weeks 60–84 of smoking, i.e., approximately the fourteenth through nineteenth months of cigarette smoking. Reported means are geometric except for smoking behavior, food intake, and water intake, for which arithmetic means are given.

Results

Cigarette Smoke Dosimetry

Smoking Behavior

The smokers averaged 471 criterion puffs per day and the shams averaged 447. The smokers received an average of 42.9 cigarettes per day, and the sham smokers were given access to shams 42.7 times a day. The overall mean puff duration requirement for criterion puffs was 2.89 seconds for smokers and 2.80 seconds for shams. No significant differences between smokers and shams were observed for mean puff volume (35.3 ml for smokers and 39.0 ml for shams); mean static pressure (6.58 cm water for smokers and 6.91 cm water for shams); mean puff duration (3.00 seconds for smokers and 3.56 seconds for shams); and criterion puffs per cigarette (10.8 for smokers and 10.6 for shams). Smokers and shams also did not differ significantly on the four PVAM variables when all puffs, both criterion and noncriterion, were considered.

Absorption of Smoke Components

The smoking animals had approximately three times more carbon monoxide and thiocyanate in their blood than the shams (table 2). The concentration of cotinine in urine was approximately six times greater in smokers than in shams, and the total daily cotinine excretion was approximately 17 times greater. All four differences were statistically significant (p < 0.01). The mean blood levels of cadmium, zinc and lead in the smokers were 0.088, 714, and 2.07 μg/dl, respectively, and the mean values for the shams were 0.101, 718, and 1.97 μg/dl, respectively. None of these differences was statistically significant.

Responses to Cigarette Smoking

Weight

The mean weight of the smokers increased from 14.9 to 17.6 kg, and the mean weight of the shams increased from 16.6 to 20.4 kg. Such weight increases were appropriate for baboons of their ages. The mean body weight and the mean linear trend in body weight over time were greater (p < .10) in shams than in smokers. However, using body weight in three weighings before smoking as covariates, the groups were not significantly different.

Food and Water Intake

The smokers consumed 315 g of food and 488 ml of water daily, and the shams consumed 350 g of food and 520 ml of water daily. Differences in both food and water intake were statistically significant (p < .05). However, when corrected for body weight, the food and water intake of smokers and shams did not differ significantly. The mean urine volume per 24 hours and mean urinary specific gravity for smokers (206 ml at 1.037 sp. gr.) and shams (186 ml at 1.038 sp. gr.) were almost identical.

Serum Lipids and Lipoproteins

The mean serum total cholesterol concentration of smokers was 5% higher than that of the shams, and the mean triglyceride concentrations were similar (table 3). The mean VLDL + LDL cholesterol concentration of smokers was 18% higher than that of the shams, and the mean HDL cholesterol concentration of smokers was 6% less than that of shams. With the exception of HDL cholesterol (p < 0.10), smokers did not differ significantly from shams on these measures.

Blood glucose

The mean fasting blood glucose concentration of smokers (table 3) was 12% higher than that of shams (p < 0.05).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Geometric mean</th>
<th>Estimated ratio</th>
<th>95% confidence limits on ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smokers</td>
<td>Shams</td>
<td>smoker/sham</td>
</tr>
<tr>
<td>Carbon monoxide (ml/dl)</td>
<td>0.24</td>
<td>0.08</td>
<td>3.08</td>
</tr>
<tr>
<td>Thiocyanate (μg/ml)</td>
<td>4.80</td>
<td>1.66</td>
<td>2.89</td>
</tr>
<tr>
<td>Urinary cotinine output (μg/24 hr)</td>
<td>775</td>
<td>45</td>
<td>17.13</td>
</tr>
<tr>
<td>Urinary cotinine concentration (μg/ml)</td>
<td>3.49</td>
<td>0.62</td>
<td>5.66</td>
</tr>
</tbody>
</table>
Erythrocytes

The mean red blood cell count was 5.13 million/mm³ in smokers and 5.11 million/mm³ in shams. The mean hemoglobin concentration was 12.9 g/dl in smokers and 12.8 g/dl in shams. The mean hematocrit was 38.8 in smokers and 38.6 in shams. None of these differences was statistically significant.

Platelets

The mean platelet count in smokers was 289 thousand/mm³ and that in shams was 278 thousand/mm³. The differences between smokers and shams in platelet count; in the slope, maximum transmittance, and lag time of the transmittance curves; and in a qualitative assessment of disaggregation were not significant.

Leukocytes

The mean leukocyte count (table 4) of the smokers was 18% higher than that of the shams (p < 0.01). Differential cell counts indicated that this difference was attributable to increased circulating lymphocytes in smokers. The mean lymphocyte count of smokers was 38% higher than that of shams (p < 0.01). None of the other leukocyte types counted differed appreciably between smokers and shams.

Discussion

Comparison of Human and Baboon Smoking Dosimetry

Puffing Behavior

In a study including quantitative measures of smoking, 500 human smokers made an average of 9.1 puffs per cigarette on 21.2 cigarettes per day. The mean puff duration was 2.13 seconds, and average puff volume was 43.2 ml, for a total daily dose of about 8.3 l of smoke. The corresponding figures for the smoking baboons in this study were 11.0 puffs per cigarette on 42.9 cigarettes per day. The mean puff duration was 3.60 seconds, and mean puff volume was 35.3 ml. The total daily dose of smoke was, therefore, about 16.6 l. Thus the average puffing behavior of the cigarette-smoking baboon was similar to that of the average human smoker.

Carbon Monoxide

In a large epidemiologic study, mean carboxyhemoglobin concentrations of 4.7% were found in “inhaling” smokers, 2.2% in “noninhaling” smokers, and 0.9% in nonsmokers. Converting the values reported here from ml CO/dl blood to percent carboxyhemoglobin, the arithmetic mean COHb values for smoking and nonsmoking baboons were 1.9% and 0.6%, respectively. Human cigarette smokers had a

<table>
<thead>
<tr>
<th>Serum component</th>
<th>Geometric mean (mg/dl)</th>
<th>Estimated ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smokers</td>
<td>Shams</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>171.6</td>
<td>162.9</td>
</tr>
<tr>
<td>Total triglyceride</td>
<td>41.3</td>
<td>41.2</td>
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<tr>
<td>HDL cholesterol</td>
<td>83.0</td>
<td>88.1</td>
</tr>
<tr>
<td>VLDL + LDL cholesterol</td>
<td>78.3</td>
<td>66.4</td>
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<tr>
<td>Fasting glucose</td>
<td>94.1</td>
<td>84.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Geometric mean (10⁶ cells/mm³)</th>
<th>Estimated ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Smokers</td>
<td>Shams</td>
</tr>
<tr>
<td>Leukocytes, total</td>
<td>7.59</td>
<td>6.42</td>
</tr>
<tr>
<td>Polymorphonuclear Leukocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Segmented</td>
<td>1.43</td>
<td>1.78</td>
</tr>
<tr>
<td>Stab</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.18</td>
<td>0.17</td>
</tr>
<tr>
<td>Basophiles</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.07</td>
<td>0.08</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>5.56</td>
<td>4.03</td>
</tr>
</tbody>
</table>
two- to fivefold elevation in blood carboxyhemoglobin, depending on inhalation pattern, over nonsmokers, whereas the cigarette smoking baboons had a threefold elevation in blood carboxyhemoglobin. The blood carboxyhemoglobin concentrations of cigarette smoking baboons correspond to the lower end of the distribution of absolute values reported for human cigarette smokers.

Nicotine and Cotinine

Cotinine, the major metabolite of nicotine, is used to measure absorption of nicotine from cigarette smoke because the parent compound is detoxified rapidly. Since nicotine absorption in the mouth is poor at the pH of cigarette smoke, and nicotine abstraction in noninhalers is much less than that of inhalers, cotinine excretion appears to be a good indicator of inhalation of cigarette smoke. A survey of human cigarette smokers found an average daily urinary excretion of 880 μg of cotinine; the mean cotinine concentration in the urine was 0.55 μg/ml. The cigarette-smoking baboons in this study averaged (arithmetic mean) 939 μg/24 hours and a mean concentration of 4.66 μg/ml.

Thiocyanate

Mean serum thiocyanate concentrations of 5.2 μg/ml in male cigarette smokers and 1.7 μg/ml in male nonsmokers have been reported. Our baboons averaged (arithmetic mean) 5.1 μg/ml for smokers and 1.7 μg/ml for shams, concentrations almost identical to those of corresponding humans.

Conclusion

Three chemical dosimetry methods, including measures of both gas and particulate phases of cigarette smoke, indicate that the baboons puffing on lighted cigarettes inhaled cigarette smoke and absorbed smoke components.

Comparison of Human and Baboon Responses

Serum Lipid and Lipoprotein Concentrations

Surveys of human smokers and nonsmokers have yielded conflicting results on serum total cholesterol concentrations; in about half, there was no difference, and in the other half, higher levels were found in smokers. As in humans, smoking had no effect on serum triglycerides in baboons. In most of the recent surveys in which lipoproteins were measured, human smokers have had lower HDL cholesterol concentrations and higher LDL cholesterol concentrations. In our experiment the effect of smoke inhalation on serum lipids and lipoproteins remains equivocal. The smokers had slightly higher serum total cholesterol and VLDL + LDL cholesterol concentrations and slightly lower HDL cholesterol concentrations than did nonsmokers, but the differences were not statistically significant. The differences are in the direction that would be expected to accompany the augmentation of atherosclerosis and may increase with continued exposure. In our baboons, smoking for 14–19 months does not appear to affect serum lipid and lipoprotein concentrations.

Leukocytes in Peripheral Blood

Leukocytosis in human smokers has been recognized in many surveys. In most studies in which differential counts were performed, both polymorphonuclear and mononuclear cells were increased. Two recent reports described lymphocytosis. The lymphocytes in both smokers and nonsmokers were predominantly T cells, and the reactivity of lymphocytes to phytohemagglutinin was increased in smokers younger than 40 years of age or with a 20 pack-year history or less of cigarette consumption. All comparisons were based on persons who had smoked much longer than the 14 months of this experiment. We could not find any reports of differential leukocyte counts in adolescent smokers who had just begun smoking.

The lymphocytosis in our smoking baboons, together with the lymphocytosis in human smokers, suggests an immunologic response to smoke inhalation. Numerous experiments with rodents have shown that short-term exposure to very high levels of tobacco smoke depresses systemic antibody responses and T-lymphocyte functions; exposure to lower smoke doses initially stimulates, and later depresses, these same responses. Several nonspecific serological abnormalities have been observed in human smokers. Based on both the animal and human studies, it appears that human-like levels of cigarette smoke exposure at first stimulate T-lymphocyte-dependent functions and later depress these functions.

The relevance of immunologic disorders to atherosclerosis is supported by the observations that immune complex disease augments atherogenesis in the rabbit and the baboon. The immunologic response to sperm antigens that often accompanies vasectomy may also exacerbate atherosclerosis. The presence of highly antigenic substances in tobacco and in cigarette smoke has been demonstrated. Because immune responses (such as altered lymphocyte functions, changes in immunoglobulin, complement, and acute phase reactant concentrations, and formation of immune complexes) may be important mechanisms by which cigarette smoking affects atherosclerotic disease, observations on these measures should be made in animal models and humans.

Platelets

We found no effects of cigarette smoking on platelet count or platelet aggregation. Surveys of human smokers have shown no change in platelet counts, but have shown differences in platelet aggregation and platelet turnover. If platelets are affected by smoking, longer or heavier smoke exposure, larger experiments, or more precise tests will be required to detect the effect.
Fasting Blood Glucose

The clearly elevated fasting blood glucose concentration in smoking baboons is consistent with reports that blood glucose is elevated in human smokers. The issue of whether hyperglycemia is responsible for the augmentation of atherosclerosis in diabetics is controversial; however, if chronic elevation of blood glucose is atherogenic, as suspected, this response to smoking also may represent an important intervening variable.

Conclusion

Using an animal model where random assignment to smoking or nonsmoking conditions and experimental control over other variables is possible, we avoided many of the possible confounding effects present in studies of responses to cigarette smoking in humans. The results available after 14 months of smoking confirm that cigarette smoking is associated with increases in lymphocyte count and fasting blood glucose concentration. However, we did not detect effects on serum cholesterol, triglyceride or lipoprotein cholesterol concentrations; platelet count and platelet aggregation; erythrocyte count; hemoglobin concentration; or hematocrit. The ability to conduct controlled studies in a human-like animal model provides new opportunities to study the ways by which cigarette smoking aggravates atherosclerosis.

Acknowledgment

Dr. A. W. Spears, Director, Research and Development, P. Lorillard Company, prepared the cigarettes used in this investigation according to the authors' specifications and analyzed the tobacco. Dr. M. R. Guerin, Analytical Chemistry Division, Oak Ridge National Laboratory, analyzed the smoke.

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