Cigarette Smoking, Air Pollution, and Immunity: a Model System

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BALB/c mice were exposed to fresh cigarette smoke, a mixture of SO₂ (5 ppm) and CO (50 ppm), or both for periods up to 18 weeks. After varying exposure times, animals were intratracheally inoculated with 10⁷ sheep erythrocytes and sacrificed 7 days later, during which time the various exposure regimes were continued. Plaque-forming-cell responses were measured in spleens and a pool of the cervical and mediastinal lymph nodes, together with serum hemagglutinating and hemolytic responses, and compared with those of age-matched control animals. Both the organ and serum responses exhibited stimulation in the early phase of exposure, before a depression with prolonged exposure. The greatest depression was seen in animals that had been chronically exposed to both fresh cigarette smoke and the gas mixture.

Cigarette smoke inhalation has been implicated in the etiology and pathogenesis of a number of respiratory diseases in man (12), and recently attention has been drawn to the possible role of immune suppression in the development of some of these diseases (9). Scattered information in the literature (4, 5, 7, 8, 11, 16) indicates that the immune system in man may be affected to some degree by smoking, and chronic inhalation experiments in this laboratory using small animals have clearly shown progressive depression of both humoral and cell-mediated immunity during exposure (13-15).

The inhalation of industrial air pollutants may also play a role in the development of respiratory disease (1, 2, 6), and recent evidence from an animal model system has also implicated damage to immunity in the development of respiratory disease in this situation (17).

A number of epidemiological studies have suggested that smoking and air pollution may act synergistically in the promotion of respiratory disease (e.g., 1, 2). In the experiments reported below, we have established a model system to investigate the combined effects of cigarette smoke and industrial air pollutants on the serum antibody and organ plaque-forming-cell responses of mice.

MATERIALS AND METHODS

Exposure techniques. BALB/c mice were divided into four equal numerical groups, matched with respect to age and sex, at the commencement of the experiment. The first group, controls (C), were maintained in the animal house. The second group (S) was exposed to fresh cigarette smoke on weekdays in the Hamburg II (Heinr. Borgwaldt, West Germany) small-animal smoking machine, set to deliver cigarette smoke mixed with air in the ratio of 1:7 (13). The daily exposure time was 7 to 8 min, equivalent to one cycle of the machine. The third group (G) was exposed to a mixture of two gases for 3 h per weekday. The gas exposures were carried out in an airtight perspex box (10 by 30 by 50 cm; 40 animals at any one time) equipped with an inlet port at one end and an outlet at the other. Sulfur dioxide at a concentration of 5 ppm and carbon monoxide at a concentration of 50 ppm, mixed with medical air, were passed through the chamber at a flow rate of 5,000 cm³/min. The gas levels used here are internationally accepted as "safe" for an 8-h occupational exposure. The gases were prepared and analyzed by Commonwealth Industrial Gases of Australia. The last group (SG) was exposed daily for 3 h to SO₂ and CO, before exposure to fresh cigarette smoke at the dosage above, 3 to 4 h later.

Immunological techniques. At various times during the exposure period, samples of mice from the four groups were inoculated intratracheally with 10⁷ sheep erythrocytes (SRBC) in 10 aliters of phosphate-buffered saline. After 7 days (determined previously as the peak of the primary humoral immune response), during which the various exposure regimes were continued, the mice received an overdose of sodium pentobarbitone and were exsanguinated. The cervical and mediastinal lymph nodes in individual animals were pooled. Cell suspensions from lymph nodes and spleens were prepared in Eagle minimal essential medium after mincing, filtration through nylon wool, and centrifugation at 120 x g for 10 min. Serum was collected from the blood samples, heated at 56 C for 30 min, and stored overnight at 4 C.

The Cunningham modification of the Jerne plaque technique (3) was used to locate direct immunoglobul-
lin (Ig) M and indirect IgG/IgA plaque-forming cells (PFC) in the lymph node and spleen samples, as previously described (13).

The results were determined as PFC/10⁴ leukocytes and converted to geometric means ± standard errors. Student’s t test was used to assess the differences between controls and exposed groups. The data were normalized (control values set at 100 PFC/10⁴ leukocytes) to facilitate graphic presentation (see figures).

To measure circulating hemaglutinating antibody, 10 μl of doubling serum dilutions (in phosphate-buffered saline) was added to 10 μl of 0.25% SRBC. Results were read against appropriate controls after incubation at 37 C for 1 h. Hemolytic antibody was titered by adding 10 μl of doubling serum dilutions to 10 μl of guinea pig complement and 10 μl of 1% SRBC. Results were again read after a 1-h incubation at 37 C. The geometric mean and standard deviation were obtained by converting the titers to logarithmic values as for PFC counts. The logarithm of the control values was then set at 1.0, and other values were expressed as a relative proportion of the controls.

RESULTS

PFC responses in normal animals. Seven days after the intratracheal inoculation of 10⁸ SRBC, spleen preparations from the control animals contained on average 105 ± 10 (geometric mean ± standard error) direct PFC/10⁴ leukocytes and 8.2 ± 2.5 indirect PFC/10⁴ leukocytes. The pooled lymph node preparations contained 80 ± 6 direct PFC/10⁴ leukocytes and 5.0 ± 2.8 indirect PFC/10⁴ leukocytes. Age-matched controls were used throughout the experiment, and no age-dependent variation was found in the respective PFC responses of the animals. Leukocyte preparations from the organs of unimmunized mice contained 0.2 to 1.3 PFC/10⁴ cells.

Relative spleen responses in test groups. Figure 1 shows the effect of chronic cigarette smoke and/or pollutant gas inhalation on the direct and indirect PFC response in the spleens of BALB/c mice. Over the periods studied, animals in group S exhibited increased direct and indirect PFC responses. The experiments using group G were terminated at week 12, when the animals were fatally overdosed due to machine malfunction, and consequently observations are reported only at weeks 5 and 10. This group exhibited an increased direct PFC response on week 5 followed by a decrease by week 10. The indirect response was slightly enhanced on both occasions. In group SG, the direct response showed the early rise before a depression. By week 18, the direct response in this group was less than 50% of that in control animals. Indirect PFC response in this group remained elevated throughout the experiment, though the increases noted were not statistically significant.

Relative lymph node responses in test groups. In Fig. 2, the direct and indirect PFC responses in the pooled cervical and mediastinal lymph nodes of the mice are shown up to 18 weeks of exposure. In group S, both the direct and indirect responses showed a biphasic pattern of change, but by week 18 both responses fell within normal limits. Up to the 10th week, group G exhibited a similar pattern of change.

Fig. 1. Direct and indirect PFC responses in spleens. a, b, c, and d represent observations on weeks 5, 10, 14, and 18, respectively. All groups were sampled at this time with the exception of group G (weeks 5 and 10 only). Data shown are the geometric mean ± standard error derived from five to ten observations. The significance of differences between control and exposed animals was determined by Student’s t test: (○) P < 0.05; (●) P < 0.01; (▲) P < 0.001.
The animals in group SG, however, exhibited a more extreme reaction to the exposure. By week 5, both the direct and indirect responses showed a massive rise. This was followed by a sharp decline, and by week 18 both the direct and indirect PFC responses were almost completely ablated. Over the 18-week period studied, there was significant correlation between the direct PFC values in the spleen and lymph nodes (P < 0.05, using product moment intercorrelation). The pattern of changes in the direct and indirect PFC responses in the lymph nodes were also significantly correlated (P < 0.05, unlike corresponding direct and indirect responses in the spleens).

**Humoral antibody responses.** The alterations noted above in the PFC responses in the spleen and lymph nodes were reflected to a large degree in the changes in humoral antibody responses (Fig. 3). In groups S and G an initial rise occurred at week 5, followed by a decline. These fluctuations, however, were not statistically significant. Group SG exhibited no initial stimulation but instead showed a steady decline in titers of both hemolytic and hemagglutinating antibody. By the 18th week, titers of both antibodies showed a significant depression and were less than half of those seen in the controls. A significant correlation was noted between the hemagglutinating and hemolytic antibody titers (P < 0.001) and between these antibody titers and the direct PFC response in the spleen (P < 0.001). This may be expected since the humoral antibody titer is usually determined by the antibody-producing cell population in the spleen (10).

**DISCUSSION**

Previous reports from this laboratory have indicated that chronic exposure of C57 Black mice to fresh cigarette smoke produces biphasic changes in humoral immune responsiveness after the intratracheal administration of immunogen (15). The suppression of immune responsiveness did not appear to result simply from depressed absorption of immunogen through the respiratory tract, since antibody production at a distant site, the spleen, was less sensitive to the effects of cigarette smoke than sites associated with the respiratory system (13).

In other experiments, biphasic changes were also noted in cell-mediated immune responsiveness of mice during exposure to cigarette smoke (14). In both the above studies, a stimulation of immune responsiveness was seen in the early stages during exposure, before an ultimate depression of responsiveness by about the 30th week of exposure. Essentially similar results have been reported by Zarkower (15), using carbon and SO2 exposure, indicating that short-term inhalation studies of the type often reported in the literature may give a completely false picture of the long-term effects.

Unlike our previous studies (15), in which the exposure period was up to 42 weeks, the present experiments were conducted over an 18-week period. In group S, the changes in the PFC responsiveness in both spleen and lymph nodes closely paralleled those previously reported over the same time scale for C57 Black mice (15). The short-term observations on group G animals indicate a similar early stimulation.
It was not possible to follow this group (G) through to 18 weeks, and consequently we can draw no meaningful comparisons between the relative effects of cigarette smoke and gases on the immune system. However, our results clearly show that joint exposure to these agents produces more marked effects on organ PFC responses and serum antibody responses than exposure to cigarette smoke alone. The double exposure produces significant immune depression with 18 weeks, whereas cigarette smoke alone may take up to 30 weeks to produce a similar effect (15).

Although preliminary, these results suggest that industrial air pollutants may exert marked effects on the immune system in situations where the added stress of cigarette smoke inhalation exists concurrently. This possibility requires further vigorous investigation, since it has serious ramifications in terms of the methods used to ascertain safety standards pertaining to occupational exposure to air pollutants; viz., "safe" exposure levels for nonsmokers may potentially exert serious effects on smokers.

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LITERATURE CITED