FINAL REPORT

ARB Contract A0-106-32

NEW APPROACH FOR DETECTING HEALTH HAZARDS OF NO_2 INHALATION

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1. ABSTRACT

Studies were carried out to determine if the effects of ambient level NO_2 inhalation which lead to facilitation of blood borne cancer cell metastasis to the lungs, were dependent upon the NO_2 exposure conditions. In all of our previous studies the pollutant exposures varied and were carried out before the introduction into the blood stream of cancer cells. In contrast, all experiments described in this report had the same NO_2 level (0.4 ± 0.05 ppm) but the exposure length and the timing, with respect to circulating cancer cells, varied. Usually, there were three groups of animals in each experiment; filtered air control, NO_2 exposed and ambient air group. The ambient air group was always kept in vivarium room air while the clean air control and the NO_2 exposed groups were kept in special environmental chambers within their respective environments, The NO_2 levels were monitored continuously. B16 mouse melanoma cells were infused intravenously into the blood of animals before or after an appropriate exposure and the incidence of metastases development, determined three weeks later, permitted the assessment of the effects.

The results have indicated that ambient level NO_2 exposures for ten to 12 weeks facilitate blood borne cancer cell metastasis to the lungs and indicate harmful NO_2 action. The NO_2 exposures for three weeks, while cancer cells are in circulation, or for six weeks before cancer cell infusion, did not show facilitation of metastasis. The introduction of clean air periods between NO_2 exposure episodes to some degree reduced the NO_2 effects but only in the less sensitive portion of the test animals. Importantly, the polluted ambient air inhalation for as short a period as six weeks, resulted in facilitation of metastasis.

These findings support our earlier studies and indicate that certain ambient level NO_2 and polluted ambient air exposures are harmful and play a role in facilitation of blood borne cancer cell metastasis to the lungs. These findings have a direct bearing on the human cancer problem since the probability exists that one in four individuals will develop cancer and most of the cancer patients have circulating cancer cells. Of primary importance is the fact that urban communities are exposed to similar levels of air pollutants almost daily. Thus the observations discussed here provide strong support for the need of improved air quality in urban areas and the need for studies to identify other air pollutants which may have the same action. The mechanisms involved remain unclear and need to be investigated. In addition, the findings should assist in establishing the rationale for setting air quality standards.

2. ACKNOWLEDGEMENTS

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3. DISCLAIMER

"The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their source or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products."

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6. SUMMARY AND CONCLUSIONS

The major objective of this study was to determine if certain exposure conditions at a constant ambient level of NO_2 (0.4 ± 0.05 ppm) could diminish or show less harmful NO_2 inhalation effects. In order to test this, experimental animals (mice) were exposed to 0.4 ± 0.05 ppm of NO_2, while the control and the ambient air animals inhaled NO_2-free or ambient Vivarium room air, respectively. Either directly following or before the exposure periods, cancer cells (B16 mouse melanoma cells) were infused into the blood stream of the animals and three weeks later the lungs were examined for the development of cancer nodules. It was reasoned that any damage incurred by inhalation of NO_2 or polluted ambient air to the lungs, to the immune system, to the blood clotting system or to a combination of these, should cause increased blood borne cancer spread or metastasis to the lungs of exposed animals. Thus, the incidence of metastases in the lungs when compared between control and exposed animals would serve as an indicator for harmful NO_2 effects. The end result, which is cancer cell metastasis, is a very meaningful biological event and has direct relevance to human health since the polluted air is encountered almost daily by city dwellers and urban workers. Most importantly, the probability exists that one in four individuals will develop cancer during their lifetime and cancer cells are found in peripheral blood in most of the cancer patients.

The results from the experiments performed have indicated that the animals exposed for ten to twelve weeks to 0.4 ± 0.05 ppm of NO_2 developed a significantly higher number of cancer nodules in their lungs when compared to lungs of filtered air controls. The finding that a six-week exposure to the same NO_2 concentration did not show facilitation of metastasis but that the same length of exposure to polluted ambient air showed

significant facilitation of metastasis, is of considerable interest. Considering that most of the time the vivarium ambient air has levels of NO_2 lower than 0.1 ppm, the observed results may be due to a combination of several low concentrations of pollutants present or a single pollutant other than NO_2. The other exposure condition which did not show metastasis facilitation was a three-week exposure immediately after cancer cell infusion and without previous NO_2 exposure. The lack of metastasis facilitation in the shorter NO_2 exposure periods, of course, does not mean that there were no effects on the host. It means that there were no changes which would facilitate blood borne cancer cell metastasis.

The other finding of interest is that introduction of a one week clean air episodes between NO_2 exposures apparently permitted some recovery or that the NO_2 effect was not as severe in the less sensitive portion of the test population. The highly sensitive portion of the population, revealed by upper quartile analysis, still showed a significantly increased incidence of metastases in both the NO_2 and the ambient air groups of animals.

Another experiment revealed that the mortality rate from progression of metastases is significantly increased in subpopulations of NO_2 and ambient air exposed animals. We interpret that these subpopulations represent animals which are most sensitive to air pollutants. Sensitivity differences to air pollutants are well recognized among humans.

Finally, the demonstration in these and our earlier experiments, that inhalation of certain ambient concentrations of NO_2 or polluted ambient air, facilitated blood borne cancer cell metastasis to the lungs, provides strong indications for the harmful action of ambient levels of NO_2 and possibly other air pollutants. It should be noted that

metastasis, the indicator for harmful effects in this system, is also the major clinical concern in cancer progression. We believe that the findings lend strong support for the need of improved urban air quality and a decrease in noxious ambient air pollutants. The mechanisms involved in facilitation of metastasis remain unknown and studies are needed to elucidate them and to identify other air pollutants which may show the same function. The findings should be of assistance in developing a rationale for setting air quality standards.

7. RECOMMENDATIONS

We have clearly demonstrated that inhalation of ambient level NO_2 plays a role in facilitation of blood borne cancer cell metastases and thus shows harmful NO_2 action. We have also shown that as short as six weeks of exposure to polluted ambient air will enhance blood borne cancer cell metastases and that clean air episodes between ambient level NO_2 exposures may diminish the NO_2 effects, at least in the less sensitive population. Thus, the results discussed in this report reinforce our previous findings and provide more support for the need of improved air quality. However, the big question still remains: is this happening in the human urban population? The answers are not available at the present time and specific epidemiological studies dealing with metastasis development and recurrence could be of assistance. The other pertinent questions are: 1. What other air pollutants may act as facilitators of cancer cell metastasis; 2. Do air pollutants facilitate primary cancer growth and dissemination, and 3. What mechanisms are involved in air pollutant facilitation of metastasis or growth of a cancer. Our studies have indicated that the above questions represent the general areas which need intensive investigation in order to determine the full impact of the hazards associated with inhalation of air pollutants and for the development of a better rationale for setting air quality standards.

8. BODY OF REPORT

Introduction

a. Scope and purpose of the project, general background of the project.

The presence of pollutants in the environment, especially those with carcinogenics properties has been of great concern to environmental health scientists. In view of this, many studies have been directed toward the identification of cancer causing agents in the environment (1-4). However, the problem of cancer involves not only the development and presence of neoplastic cells at a primary site, but also the ability of these cells to migrate, seed, proliferate and develop secondary cancer masses or metastases in distant organs and tissues (5,6). Considering the fact that a significant segment of the population in the United States is already affected by cancer together with the probability that one in four individuals will develop cancer (7), the question arises as to the role environmental pollutants play not only in the causation of cancer or carcinogenesis, but in the dissemination of cancer cells and the development of metastases. It is well established that most cancer patients have circulating cancer cells (8,9) and in some instances cancer cells have been demonstrated in the circulation of patients who are clinically free of cancer (10,11). Circulating cancer cells are also found in the peripheral blood of tumor bearing animals (12). Moreoever, there are several known conditions which may favor the development of cancer cell metastases from circulating cancer cells and include the following: 1) immune suppression, 2) capillary endothelial cell alterations, 3) cancer cell interactions with components of blood clotting mechanisms and 4) tissue damage in general (4,5,13-17). Importantly, it is known that several of the above mentioned conditions occur as a result of nitrogen dioxide (NO_2), a common air pollutant, inhalation (18-21) and thus one may expect

that NO_2 inhalation could facilitate or enhance circulating cancer cell metastasis. Most pertinent to this project are the studies showing changes in lung capillaries and in the cells of the lung itself due to NO_2 exposure (22-24) since the capillary cell injury and trauma in general have been associated with enhanced blood borne cancer cell lodgement, proliferation and metastases development (11,15,25).

Keeping in mind the foregoing, we designed studies to determine if NO_2 would act as a facilitator for blood borne cancer cell metastasis to the lungs. By comparing the incidence of lung metastases development in exposed and control animals the adverse NO_2 effects would be detected. The experiments utilized B16 mouse melanoma cells as a model. To the best of our knowledge these are the only studies where blood borne cancer cells have been used as a probe to detect adverse ambient level NO_2 effects.

The results of these experiments indeed have demonstrated that intermittent inhalation of 0.3, 0.4, or 0.8 ppm of NO_2 for a period of ten to 12 weeks, facilitated blood borne cancer cell metastasis to the lungs of exposed animals (26, 27,28). In addition, animals inhaling polluted Los Angeles ambient air (vivarium room air) also developed significantly greater number of melanoma nodules in their lungs than the controls. It is important to point out that NO_2 exposures in our earlier studies were always before the cancer cell infusion into the blood stream. In contrast, the experiments carried out under the present Contract had constant NO_2 level (0.4 ± 0.05 ppm) but the exposure schedule varied. There were five different experiments, each designed to test significantly different exposure condition which may influence the incidence of lung metastasis development and that way would indicate diminished or enhanced NO_2 effects. Moreover, these studies have a direct bearing on the human health because the same or very similar conditions may exist in many urban populations. The increased cancer

mortality in urban areas, as reported by some investigators (29,30), may mean increased metastasis. It is also of interest that increase in lung metastases from subcutaneous melanoma implants has been observed in mice exposed to cigarette smoke (31).

b. Design, Materials and Methods

An Overview

The experiments described in this report utilized the same B16 mouse melanoma system which we employed in previous studies. Five-week old C-57 BL/6J male mice were used in all experiments since the B16 melanoma originated in this strain of mice and thus provided a syngeneic system (32). In all experiments the NO_2 level was kept at 0.4 $\pm$ 0.05 ppm but the exposure length and frequency was varied for each specific experiment as described below. The NO_2 and the clean air exposures were carried out in special identical environmental chambers while the ambient group animals were kept in vivarium room air. The desired NO_2 concentration was delivered to NO_2 exposure chambers according to the method developed in this laboratory (33) and the NO_2 concentrations were monitored by Saltzmans (34) and chemiluminescence methodologies. Either following or before the appropriate exposure, animals of all groups were infused intravenously with 10^5 viable melanoma cells. The melanoma cells were carried in tissue culture and single cell suspensions were prepared when needed for infusion. For the next three weeks the NO_2 exposed and the clean air animals were kept in filtered clean air environment while the ambient air group remained in an ambient air environment.

The surviving infused cells developed black malanoma nodules in the lungs, which can be easily counted on acetate buffered formalin perfused gross specimens of the lungs using a stereo microscope. By counting the nodules at 21 to 23

days after intravenous infusion of the melanoma cells quantifiable data can be obtained. However, if the nodules were permitted to grow the animals died from metastases four to seven weeks later. By comparing the incidence of melanoma development in the lungs of NO_2 exposed, ambient air exposed, and the control animals (clean air exposed), the role of polluted ambient air or the NO_2 and the expsoure condition in developing host alterations and facilitation of metastases could be assessed. Limited histological studies of lungs were also carried out for all experiments.

Specific Experiments

Experiment M-144 - 3 weeks of 0.4 ± 0.05 NO_2 exposure

This experiment was designed to determine the incidence of blood borne cancer cell metastases development in lungs of animals who harbor circulating cancer cells and encounter ambient level (0.4 ± 0.05 ppm) NO_2 exposure for a period of three weeks (5 days/week, 7 hrs./day) for a total of 121 hrs. There were two groups of animals: the controls and the NO_2 exposed. Each group contained 30 animals. Both groups of animals were infused with melanoma cells and were placed immediately into their respective environmental chambers. One animal in the control group died during the cell infusion procedure. This was the first time that this type of an experiment was performed. All previous experiments in our laboratory involved animal exposure to NO_2 before the cancer cell infusion into the circulation. It was a short exposure, since animals developed lung metastases after two to three weeks and if permitted to live began to die after five weeks. For the foregoing reasons, the animals were sacrificed 23 days after the cancer cell infusion which is the optimal time for quantitation of cancer cell nodules. The bronchial tree of the lungs were perfused with acetate buffered formalin and the nodules were counted using a stereo microscope. The data were evaluated according to the Mann-Whitney statistical test and are presented in the Result section - Table 1.

Experiment M-145 - 6 weeks of 0.4 $\pm$ 0.05 ppm NO_2 exposure

This experiment was designed to determine if six weeks of NO_2 exposure (5 days a week, 7 hours a day for a total of 206 hours) at 0.4 $\pm$ 0.05 ppm before cancer cell infusion into circulation will induce facilitation of blood borne cancer metastasis to the lungs. There were three groups of animals: 1. the control with 30 animals; 2. the NO_2 exposed with 30 animals, and 3. the ambient air group with 20 animals. During the first six weeks of the experiment one control, two NO_2 exposed and four ambient air control animals were lost due to unidentified disease processes. Thus at the end of the NO_2 exposure period there were sixteen animals in the ambient group, 29 in the clean air controls and 28 in the NO_2 exposed group. At this time all animals were infused intravenously with 10^5 melanoma cells. For the next 23 days the clean air control and the NO_2 exposed group were kept in a filtered clean air environment while the ambient air group still remained in the ambient air. Following the 23-day period the animals were terminated and the lungs were removed and fixed by perfusing the bronchial tree with buffered formalin. The lungs were then examined with stereo microscope and the melanoma nodules were counted. The results are presented in the section on Results - Table 2.

Experiment M-146 - 12 weeks of 0.4 $\pm$ 0.05 ppm NO_2 exposure

This experiment was designed to determine the influence of 12 weeks of NO_2 (0.4 $\pm$ 0.05 ppm) exposure on the incidence of blood borne cancer cell metastasis to the lungs. The NO_2 exposure was carried out as before for a total period of 420 hours. There were only two groups of animals in this experiment: the NO_2 exposed and the filtered clean air group. At the end of a 12-week exposure all animals were infused intravenously with 10^5 melanoma cells. The animals were then kept for a period of three weeks in a filtered clean air environment. After this time period the animals were sacrificed,

the lungs were perfused with acetate buffered formalin and stored in the same. The counting of melanoma nodules was carried out as before using stereo microscope. The findings are reported in the Results section - Table 3.

Experiment M-149 - Alternation of 0.4 ± 0.05 ppm NO_2 and clean air exposure

This experiment was designed to determine how repeated alternations of NO_2 (0.4 ± 0.05 ppm) and clean air periods influence the incidence of blood borne cancer cell metastasis to the lungs. All exposures were carried out before cancer cell infusion into the circulation. There were three groups of animals: 1. control animals, 2. NO_2 exposed animals, and 3. ambient air inhaling animals. With respect to NO_2 exposure there were three NO_2 exposure periods (6 weeks, 3 weeks, and 3 weeks), and each NO_2 period was followed by one week of filtered clean air. The total length of NO_2 exposure was 441 hours. Following the designed exposure all animals were infused intravenously with 10^5 melanoma cells. Three weeks later the animals were sacrificed, the lungs were perfused with acetate buffered formalin and the nodules were counted as before using a stereo microscope. The findings are reported in the section on Results Tables 4 and 5.

Experiment M-152 -12 weeks of 0.4 ± 0.05 ppm NO_2 exposure

This experiment was designed to determine how a 12-week intermittent NO_2 (0.4 ± 0.05 ppm) exposure (5 days per week, 7 ± .5 hours per day) effects the rate of mortality from metastases among NO_2 exposed, ambient air, and clean air groups of animals. There were 40 control, 40 NO_2 exposed, and 18 ambient air group animals. During the 12-week exposure periods two control and three NO_2 exposed animals were lost due to unidentified causes. At the end of 12-week exposure period all the remaining animals were infused intravenously with

10^5 melanoma cells. From this point on the control and the NO_2 animal groups received filtered clean air while the ambient group remained in the ambient environment. A daily log of surviving animals was kept for all three groups. After death the animals were autopsied and the extent of metastases was determined. The findings are reported in the section on Results.

Data Analysis

All data were analyzed by nonparametric statistic methods. The nonparametric methods have an advantage over parametrics since they do not depend upon stringent assumptions and have no set minimal sample size. The Mann-Whitney test was found to be the most suitable.

c. Results

Experiment M-144 - Three Weeks of 0.4 ± 0.05 ppm of NO_2 exposure

In this experiment the NO_2 exposure was initiated immediately after the melanoma cell infusion into circulation and animals had no previous exposure. The results are summarized in Table 1. It can be seen that NO_2 exposed mice had 36.2 melanoma nodules per lung and 7.2 nodules per lobe of the lung. The control animals had an average of 35.0 melanoma nodules per lung and 7.05 per lobe. Statistical analysis of these data did not reveal significant differences between control and exposed animal groups. It is concluded that a three-week NO_2 exposure at 0.4 ppm initiated immediately after the cancer cell infusion into the circulation and without previous NO_2 exposure, does not affect the incidence of lung metastases development.

Experiment M-145 - Six weeks of NO_2 (0.4 ± 0.05 ppm) exposure

In this experiment the NO_2 exposure was carried out before the cancer cell infusion into the circulation and there were three groups of animals: 1. NO_2 exposed, 2. clean air controls, and 3. an ambient air group. The total NO_2 exposure was 206 hours. The results are summarized in Table 2. Statistical analysis revealed no significant differences between nodules of NO_2 exposed animals and the filtered air controls. However, the ambient air group showed statistically significantly greater number of melanoma nodules in their lungs than the filtered air controls ($p<0.005$).

Experiment M-146 - 12 weeks of 0.4 $\pm$ 0.05 ppm of NO_2 exposure

In this experiment NO_2 exposure was carried out before melanoma cell infusion into circulation. The results are summarized in Table 3 and it can be seen that there was significantly greater number of nodules in the lungs of NO_2 exposed animals ($p<0.01$). Originally there were 50 control animals and 51 in the NO_2 exposure group. However, two control animals were lost during the 12-week exposure period which accounts for the different numbers of animals in the respective groups.

Experiment M-149 - Alternation of 0.4 $\pm$ 0.05 ppm of NO_2 and filtered clean air exposures

The total period of NO_2 exposure in this experiment was 441 hours but the six-week and each of the three-week NO_2 exposure periods was followed by one week of filtered clean air. The results are summarized in Table 4. There were 30 animals in the control and in the ambient group but 31 in NO_2 exposed. One control animal died during the 12-week exposure period. It can be seen that NO_2 exposed animals developed a greater number of nodules in their lungs than the filtered clean air controls. However, the difference is not significant. On the contrary, the ambient air group animals developed significantly greater numbers of melanoma nodules in their lungs than the filtered air controls ($p = 0.008$). Further analysis of the data utilizing the upper quartile data revealed that both groups, that is the NO_2 exposed as well as the ambient air group developed greater numbers of melanoma nodules in their lungs than the filtered clean air controls. The upper quartile analysis is summarized in Table 5.

Experiment M-152, 12 weeks of 0.4 $\pm$ 0.05 ppm of NO_2 exposure

This experiment was designed to determine the differences in survival among the NO_2 exposed animals, filtered clean air animals, and the ambient air animals. The evaluation of data revealed that the mortality rate varied within and among the experimental groups. In view of this, the data were analyzed according to time periods reflecting these variations. The survival study was divided into four time periods starting with day 28, the day before the first death occurred. The statistical summary of the percent survivors, including regression analysis and analysis of covariance, is presented in Table 11. For the first period, the daily percent mortality rate of the control group was a -4.47. The daily mortality rate for ambient air group and NO_2 exposed groups were double the control value, i.e. -10 and -9.72, respectively. The F test for the homogeneity of regression coefficient indicated a significant difference in mortality rates at the 5% level ($F = 5.96$). The analysis of covariance showed that the average percentage survival of the three groups was significantly different at the 5% level, i.e. the average survival for the control group was 89.5%, the survival of both the NO_2 exposed and ambient group was approximately 81% (Table 11 and Fig. 1). Using regression analysis, no significant differences were detected for the percent mortality rates of the combined second and third time periods. However, the analysis of covariance indicated that the percent survival of the three groups was significantly different ($F = 6.33$). Average percent survival was greater in control group (31.0%), followed by ambient air group at 24.4% and NO_2 exposed at 21.4% (Table 11).

None of the surviving ambient air and NO_2 exposed mice died in the fourth period and consequently their mortality rates dropped to 0. The sustained loss of control mice resulted in a daily decline of -2.10 percent, which was about half of the previous mortality rates. The last part of survival period (10 days) had too small a number of animals surviving in respective groups to make valid comparisons of mortality rates or survival.

Histopathology

Limited microscopic examinations of randomly selected samples from all experiments were carried out. The morphology of melanoma nodules was consistent with malignant growth, that is, it showed cell proliferation, invasion and destruction of lung parenchyma. The morphology and staining properties of the malignant cells were also consistent with those of malignant melanocytes. The identity of cells was further confirmed by electron microscopic examination of some specimens which revealed melanosomes, characteristic organelles of melanocytes, in different stages of development. The melanoma nodules showed a very limited inflammatory response, consisting of a few lymphocytes and macrophages. In animals which died from metastases the melanoma cell growth was very extensive in the thoracic cavity and in most cases filled the thoracic cavity and some of the lobes of the lungs were completely replaced by melanoma cells.

d. Discussion

The experiments carried out under this Contract utilized the same B16 mouse melanoma model as in our previous experiments, where the circulating cancer cells were used as a probe and the developing lung metastases as an indicator for adverse NO_2 effects. The NO_2 exposure schedule used in these experiments differed from our previous studies. Namely, the NO_2 concentration was kept constant at 0.4 ± 0.05 ppm, a level often observed in Los Angeles, while the exposure conditions were varied. The latter refers to the length of exposure, the timing of exposure with respect to the presence of circulating cancer cells and the alternation of NO_2 episodes with clear air periods. The main objective of these experiments was to see if the above mentioned conditions would increase or decrease the incidence of lung metastases development from blood borne cancer cells and would permit to determine the role the exposure conditions play in inducing adverse effects or alterations in the host.

In one of these experiments the NO_2 exposure began immediately after melanoma cell perfusion into the circulation. It was continued for a three week duration. The reason for the short exposure was that the perfused cells would develop extensive metastases during the fourth week and quantitation of the melanoma nodules would be impossible. The results from this experiment indicated that such exposure does not influence the incidence of metastases to the lungs (Table 1). This meant that the overall host status was not affected to the extent that it would favor the dissemination and growth of blood borne cancer cells. However, we cannot exclude the possibility that some alterations in the host may have occurred but may have been unrelated to metastases development. The other experiment where the incidence of metastases development in the lungs was not affected significantly was a six-week $0.4 \pm .05$ intermittent

NO_2 exposure, prior to melanoma cell infusion. However, the same length of exposure to polluted ambient air showed a significantly greater number ($p<0.005$) of melanoma nodules in the lungs of exposed animals (Table 2). This latter finding provides further evidence that even relatively short ambient air exposures, presumably when pollutant concentrations are high, produce alterations in the host which favor metastasis development. It should be pointed out that in Experiment M145 (Table 2) one would have expected higher number of melanoma nodules in all groups. However, variations are expected in biological system, particularly when we are dealing with living cells. For each experiment, we prepare cell suspensions from stock cultures but some unknown factors may play a role and occasionally will give different baseline with the same stock culture. We have experienced this before but we have not been able to provide definitive explanation for it.

In the experiment with an intermittent NO_2 exposure, two times as long (12 weeks) as mentioned above, the exposed animals developed significantly greater numbers ($p<0.01$) of melanoma nodules than the controls (Table 3). The latter finding falls in line with our results from a previous experiment where animals were exposed to 0.3 ppm for 12 weeks. It appears that the data from these and our earlier studies tend to indicate that in the range of 0.3 to 0.8 ppm of a NO_2 exposure, at least ten weeks of an intermittent exposure (5 days/week, 7 hours/day) is required to detect adverse NO_2 effects using this methodology, since 3, 6, and 8 week NO_2 exposures with similar levels have not produced increased incidence of lung metastases. With respect to polluted ambient air the picture is not as clear, mainly because we have not been able to monitor closely the different pollutants present in polluted ambient air. However, as short as six weeks of exposure to polluted ambient air has increased the incidence of lung metastasis development from circulating cancer cells and the longer exposures have done the same. To date, we have had only one experiment with eight weeks of ambient air exposure with negative results while in the other four experiments where the exposure was six to 12 weeks, the results have been positive. It is clear that polluted ambient air can produce adverse effects but which of the pollutants or combination of pollutants played the role can not

be determined from our experiments.

To gain a better understanding of the role the NO_2 exposure conditions play in the development of host alterations, one experiment was carried out where the NO_2 (0.4 ± 0.05 ppm) episodes were alternated with clean air periods. The results indicated that the NO_2 exposed animals developed more melanoma nodules than the controls but the difference was not significant. However, the animals inhaling polluted urban air, developed a significantly greater number of melanoma nodules (Table 4). The Mann-Whitney test is a non-parametric, conservative test and the p value of 0.075 in comparing filtered air group versus NO_2 exposed comes very close to being significant. In fact the t test for the same data gives $p<0.031$, which is significant, but since the melanoma nodule distribution data does not meet the criteria for t test, we prefer to report the Mann-Whitney results. In comparing controls versus room controls the probability using Mann-Whitney test was 0.008 and that of t test 0.0043. We feel that the significance with Mann-Whitney test is real, while t test results could be questioned on some occasions. Since there were considerable greater numbers of nodules in NO_2 exposed animals, data analysis of upper quartile was carried out to see if there would be significant differences in this portion of the animal population. The results revealed that in both groups the NO_2 and the ambient air, there was a significantly greater number of melanoma nodules in the lungs of these animals than the controls (Table 5). This indicated to us that we were dealing with animals of different sensitivity and the more sensitive animals (upper quartile) had developed more melanoma nodules. Similar sensitivity differences to pollutants are well recognized in the human population (35). Moreover, this finding also suggests that periods of clean air between the ambient level NO_2 exposures may permit partial recovery or at least reduce the severity of NO_2 effects in the less sensitive animals. The latter, if confirmed would be an extremely important finding.

In the experiment where the mortality rate was studied, the finding that NO_2 and ambient air exposed animals died at significantly higher rate during the five day period after the first death,

is another indication of sensitivity differences between animals in the same group. The more sensitive animals have developed more metastases which enhanced further dissemination and accelerated deaths. According to autopsies the death was due to extensive lung, thoracic cavity and mediastinal metastases. This is the first time where accelerated death has been observed due to cancer which metastasized subsequent to ambient level NO_2 exposure. Moreover, other investigators have observed an increased death rate from metastases, developing from transplanted cancer cells in cigarette smoke exposed mice (31). With respect to human cancers, some epidemiological studies have reported increased cancer mortality in polluted urban areas (29,30) while other studies have not found this correlation (36,37).

It is pertinent to emphasize that the findings of our studies, even if in an experimental animal system, warrants concern about inhalation of noxious air pollutants in general and its effects in human urban populations in particular. Such concern is particularly important in view of the estimation that one out of every four individuals will develop cancer during their lifetime (7) and that many cancer patients have circulating cancer cells in their blood (9). Some epidemiological studies correlating increased cancer mortality with polluted urban environment may be indicative of such events (29,30). However, specific epidemiological studies where the time and frequency of metastases occurrence have been correlated with air pollutant exposure are missing. Most importantly, the data discussed in this report provide additional support for the need of improved air quality and the reduction of ambient air pollutants. Further studies evaluating other air pollutant effects and exposure condition effects on cancer metastasis are needed.

Table 1

Incidence of Melanoma Nodules in Lungs

3 Weeks of 0.4 ± 0.05 ppm NO_2 Exposure

Treatment	No. Animals	$\bar{M}$ Nodules per Lobe	$\bar{M}$ Nodules per Lung	Mann-Whitney Probability
Filtered clean air	29	7.0	35.0	FA vs. NO_2 NS
NO_2	30	7.2	36.2	

Table 2

Incidence of Melanoma Nodules in Lungs

6 Weeks of 0.4 ± 0.05 ppm NO_2 Exposure

Treatment	No Animals	$\bar{M}$ Nodules per Lung	Mann-Whitney Probability
Filtered clean air	29	3.8	FA vs NO_2 NS
NO_2	28	4.9	
Ambient air (vivarium room air)	16	10.2	FA vs AA $p<0.005$

$\bar{M}$ - mean number

FA - filtered clean air

NS - Not significant

AA - Ambient air

Table 3

Incidence of Melanoma Nodules in Lungs
12 Weeks of 0.4 ± 0.05 ppm NO_2 Exposure

Treatment	No. Animals	$\bar{M}$ Nodules per Lung	Mann-Whitney Probability
Filtered clean air	48	35.0	FA vs NO_2 $p<0.01$
NO_2	51	50.0	

Table 4

Incidence of Melanoma Nodules in Lungs
Alternate 0.4 ± 0.05 NO_2 and Clean Air Exposures

Treatment	No. Animals	$\bar{M}$ Nodules per Lung	Mann-Whitney Probability
Filtered clean air	29	18.4	FA vs NO_2
NO_2	31	28.6	$p = 0.075$
Ambient air (vivarium room air)	30	30.7	FA vs AA $p = 0.008$

Table 5

Incidence of Melanoma Nodules in Lungs
Alternate 0.4 ± 0.05 ppm NO_2 and Clean Air Exposures
Upper Quartile Analysis

Treatment	$\bar{M}$ Nodules per Lung	Mann-Whitney Probability
Filtered clean air	36.6	FA vs NO_2 $p = 0.013$
NO_2	63.5	FA vs AA
Ambient air (vivarium air)	65.6	$p = 0.012$

$\bar{M}$ - mean number

FA - Filtered clean air

AA - Ambient air

Table 6

Daily NO_2 (0.4 $\pm$ 0.05 ppm) Exposures - Exp. M144

Date	Time ON	Time OFF	Hrs/NO_2/ Day	Remarks
6-8-81	9:20	16:40	7:20	
6-9-81	8:35	15:35	7:00	
6-10-81	9:00	16:40	7:40	
6-11-81	9:00	16:15	7:15	
6-12-81	10:00	17:10	7:10	
6-13-81				Filtered clean air
6-14-81				
6-15-81	9:35	16:40	7:05	
6-16-81	14:30	15:30	1:00	
6-17-81	10:30	16:50	6:20	
6-18-81	9:30	16:30	7:00	
6-19-81	9:45	16:45	7:00	
6-20-81				Filtered clean air
6-21-81				
6-22-81	10:20	17:20	7:00	
6-23-81	9:00	16:00	7:00	
6-24-81	10:00	17:05	7:05	
6-25-81	9:30	16:30	7:00	
6-26-81	10:05	17:00	6:55	
6-27-81				Filtered clean air
6-28-81				
6-29-81	10:25	17:20	6:55	
6-30-81	8:45	16:00	7:15	
7-1-81	10:00	17:10	7:10	
			Total hrs NO_2 - 121	

Table 7

Daily NO_2 (0.4 $\pm$ 0.05 ppm) Exposures - Exp. M145

Date	Time ON	Time OFF	Hrs/day	Remarks
6-8-81	9:20	16:40	7:20	
6-9-81	8:35	15:35	7:00	
6-10-81	9:00	16:40	7:40	
6-11-81	9:00	16:15	7:15	
6-12-81	10:00	17:10	7:10	
6-13-81				Filtered clean air
6-14-81				
6-15-81	9:35	16:40	7:05	
6-16-81	14:30	15:30	1:00	Air cond. problem
6-17-81	10:30	16:50	6:20	
6-18-81	9:30	16:30	7:00	
6-19-81	9:45	16:45	7:00	
6-20-81				Filtered clean air
6-21-81				
6-22-81	10:20	17:20	7:00	
6-23-81	9:00	16:00	7:00	
6-24-81	10:00	17:05	7:05	
6-25-81	9:30	16:30	7:00	
6-26-81	10:05	17:00	7:05	
6-27-81				Filtered clean air
6-28-81				
6-29-81	10:25	17:20	6:55	
6-30-81	8:45	16:00	7:15	
7-1-81	10:00	17:10	7:10	
7-2-81	8:45	16:10	7:25	
7-3-81	10:30	17:15	6:45	
7-4-81				Filtered clean air
7-5-81				
7-6-81	10:00	17:00	7:00	
7-7-81	9:00	16:00	7:00	
7-8-81	9:50	16:50	7:00	
7-9-81	9:00	16:00	7:00	
7-10-81	9:45	16:15	6:30	
7-11-81				Filtered clean air
7-12-81				
7-13-81	10:00	16:50	6:50	
7-14-81	9:25	17:10	7:45	
7-15-81	9:55	17:30	7:35	
7-16-81	9:10	16:15	7:05	
7-17-81	10:05	17:00	6:55	Total hrs. NO_2 - 206
↓				Filtered clean air
8-9-81				

Table 8

Daily NO_2 (0.4 ± 0.05 ppm) Exposures - Exp. M146

Date	Time ON	Time OFF	Hr./min/ day	Remarks
7-10-81	9:45	16:15	6:30	
7-11-81				Filtered clean air
7-12-81				
7-13-81	10:0	16:50	6:50	
7-14-81	9:25	17:10	7:45	
7-15-81	9:55	17:30	7:35	
7-16-81	9:00	16:50	7:50	
7-17-81	10:05	17:00	6:55	
7-18-81				Filtered clean air
7-19-81				
7-20-81	9:40	16:35	6:55	
7-21-81	7:35	15:20	7:45	
7-22-81	9:50	16:30	6:40	
7-23-81	7:55	16:17	8:22	
7-24-81	9:25	16:35	7:10	
7-25-81				Filtered clean air
7-26-81				
7-27-81	9:00	16:20	7:20	
7-28-81	8:40	16:00	7:20	
7-29-81	8:50	16:20	7:30	
7-30-81	9:15	16:35	7:20	
7-31-81	9:40	16:40	7:00	
8-1-81				Filtered clean air
8-2-81				
8-3-81	9:15	16:15	7:00	
8-4-81	9:30	16:35	7:05	
8-5-81	9:15	16:15	7:00	
8-6-81	9:35	16:30	6:55	
8-7-81	9:30	16:55	7:25	
8-8-81				Filtered clean air
8-9-81				
8-10-81	9:05	16:05	7:00	
8-11-81	9:35	17:05	7:30	
8-12-81	9:00	16:00	7:00	
8-13-81	9:20	16:25	7:05	
8-14-81	9:50	17:00	7:10	
8-15-81				Filtered clean air
8-16-81				
8-17-81	11:10	18:20	7:10	
8-18-81	9:15	16:15	7:00	
8-19-81	9:20	16:20	7:00	
8-20-81	9:50	16:50	7:00	
8-21-81	9:30	16:30	7:00	
8-22-81				Filtered clean air
8-23-81				

cont. of Table 8

8-24-81	9:20	16:30	7:10	
8-25-81	9:40	16:40	7:00	
8-26-81	10:00	16:45	6:45	
8-27-81	9:05	16:40	7:35	
8-28-81	9:10	16:00	6:50	
8-29-81				
8-30-81				Filtered clean air
8-31-81	10:00	17:00	7:00	
9-1-81	8:25	15:30	7:05	
9-2-81	9:00	15:45	6:45	
9-3-81	8:35	15:30	6:55	
9-4-81	10:00	17:00	7:00	
9-5-81				
9-6-81				Filtered clean air
9-7-81	13:00	19:05	6:05	
9-8-81	8:35	15:40	7:05	
9-9-81	9:30	16:35	7:05	
9-10-81	9:05	15:50	6:45	
9-11-81	9:45	16:35	6:50	
9-12-81				
9-13-81				Filtered clean air
9-14-81	9:14	16:40	7:26	
9-15-81	8:55	15:55	7:00	
9-16-81	9:30	16:30	7:00	
9-17-81	8:40	16:05	7:25	
9-18-81	9:20	16:20	7:00	
9-19-81				
9-20-81				Filtered clean air
9-21-81	9:40	17:05	7:25	
9-22-81	7:55	15:25	7:30	
9-23-81	9:25	16:25	7:00	
9-24-81	7:55	15:08	7:13	
9-25-81	9:45	16:55	7:10	
9-26-81				
9-27-81				Filtered clean air
9-28-81	8:50	16:10	7:20	
9-29-81	8:20	15:20	7:00	
9-30-81	8:35	15:10	6:35	
10-1-81	8:55	15:10	6:15	Total hrs. NO_2 - 420
				Filtered clean air
10-27-81				

Table 9

Daily NO_2 (0.4 ± 0.05 ppm) Exposures - Exp. M149

Date	Time ON	Time OFF	Hr/min/ day	Remarks
8-17-81	11:10	18:20	7:10	
8-18-81	9:15	16:15	7:00	
8-19-81	9:20	16:20	7:00	
8-20-81	9:50	16:50	7:00	
8-21-81	9:30	16:30	7:00	
8-22-81				
8-23-81				Filtered clean air
8-24-81	9:20	16:30	7:10	
8-25-81	9:40	16:40	7:00	
8-26-81	10:00	16:45	6:45	
8-27-81	9:05	16:40	7:35	
8-28-81	9:10	16:00	6:50	
8-29-81				
8-30-81				Filtered clean air
8-31-81	10:00	17:00	7:00	
9-1-81	8:25	15:30	7:05	
9-2-81	9:00	15:45	6:45	
9-3-81	8:35	15:30	6:55	
9-4-81	10:00	17:00	7:00	
9-5-81				
9-6-81				Filtered clean air
9-7-81	13:00	19:05	6:05	
9-8-81	8:35	15:40	7:05	
9-9-81	9:30	16:35	7:05	
9-10-81	9:05	15:50	6:45	
9-11-81	9:45	16:35	6:50	
9-12-81				
9-13-81				Filtered clean air
9-14-81	9:14	16:40	7:26	
9-15-81	8:55	15:55	7:00	
9-16-81	9:30	16:30	7:00	
9-17-81	8:40	16:05	7:25	
9-18-81	9:20	16:20	7:00	
9-19-81				
9-20-81				Filtered clean air
9-21-81	9:40	17:05	7:25	
9-22-81	7:55	15:25	7:30	
9-23-81	9:25	16:25	7:00	
9-24-81	7:55	15:08	7:13	
9-25-81	9:45	16:55	7:10	
9-26-81				
9-27-81				Filtered clean air
9-28-81	8:50	16:10	7:20	
9-29-81	8:20	15:20	7:00	
9-30-81	8:35	15:10	6:35	
10-1-81	8:55	15:10	6:15	
10-2-81				
				Filtered clean air
10-8-81				

cont. of Table 9

10-9-81	9:35	18:00	8:25	
10-10-81				
10-11-81				Filtered clean air
10-12-81	9:00	16:00	7:00	
10-13-81	8:15	15:15	7:00	
10-14-81	9:00	16:30	7:30	
10-15-81	8:15	16:25	8:10	
10-16-81	9:00	16:30	7:30	
10-17-81				
10-18-81				Filtered clean air
10-19-81	9:00	16:05	7:05	
10-20-81	9:00	16:00	7:00	
10-21-81	9:10	16:00	6:50	
10-22-81	8:55	16:25	7:30	
10-23-81	8:30	15:30	7:00	
10-24-81				
10-25-81				Filtered clean air
10-26-81	8:45	15:40	6:55	
10-27-81	8:20	15:20	7:00	
10-28-81	8:50	16:00	7:10	
10-29-81	8:30	15:30	7:00	
10-30-81				
↓				Filtered clean air
11-5-81				
11-6-81	8:55	16:30	7:35	
11-7-81				
11-8-81				Filtered clean air
11-9-81	8:00	16:05	8:05	
11-10-81	8:07	16:07	8:00	
11-11-81	8:00	15:00	7:00	
11-12-81	8:03	15:00	6:57	
11-13-81	8:14	15:05	6:51	
11-14-81				
11-15-81				Filtered clean air
11-16-81	8:09	15:29	7:20	
11-17-81	8:42	15:45	7:03	
11-18-81	8:45	15:45	7:00	
11-19-81	8:55	15:46	6:51	
11-20-81	8:30	15:50	7:20	
11-21-81				
11-22-81				Filtered clean air
11-23-81	7:58	15:30	7:32	
11-24-81	7:58	15:30	7:32	
11-25-81	8:27	15:30	7:03	Total hrs. NO_2 - 441
↓				Filtered clean air
12-7-81				Cell infusion
↓				Filtered clean air
12-29-81				

Table 10

Daily NO_2 (0.4 ± 0.05 ppm) Exposures - Exp. M152

Date	Time ON	Time OFF	Hrs/min/ day	Remarks
2-22-82	12:00	17:00	5:00	
2-23-82	7:49	15:20	7:31	
2-24-82	8:33	16:40	8:07	
2-25-82	7:50	14:55	7:05	
2-26-82	9:07	16:05	6:58	
2-27-82				
2-28-82				Filtered clean air
3-1-82	8:52	16:10	7:18	
3-2-82	7:41	15:40	7:59	
3-3-82	8:08	15:40	7:32	
3-4-82	7:45	14:45	7:00	
3-5-82	9:27	16:41	7:14	
3-6-82				
3-7-82				Filtered clean air
3-8-82	9:36	16:11	6:35	
3-9-82	7:55	15:50	7:55	
3-10-82	9:16	16:10	6:54	
3-11-82	8:00	15:50	7:50	
3-12-82	10:10	16:30	6:20	
3-13-82				
3-14-82				Filtered clean air
3-15-82	8:30	15:47	7:17	
3-16-82	7:55	16:06	8:11	
3-17-82	9:10	16:10	7:00	
3-18-82	8:30	16:10	7:40	
3-19-82	9:30	16:32	7:02	
3-20-82				
3-21-82				Filtered clean air
3-22-82	10:40	18:17	7:37	
3-23-82	8:10	15:20	7:10	
3-24-82	10:20	17:00	6:40	
3-25-82	7:20	15:20	8:00	
3-26-82	10:20	16:20	6:00	
3-27-82				
3-28-82				Filtered clean air
3-29-82	11:00	18:05	7:05	
3-30-82	7:30	15:30	8:00	
3-31-82	11:45	16:45	5:00	
4-1-82	7:35	15:08	7:33	
4-2-82	10:05	16:00	5.55	
4-3-82				
4-4-82				Filtered clean air
4-5-82	9:35	16:30	6:55	
4-6-82	7:25	15:18	7:53	
4-7-82	9:15	16:15	7:00	
4-8-82	7:20	14:30	7:10	
4-9-82	9:35	16:47	7:12	
4-10-82				
4-11-82				Filtered clean air

cont. of Table 10

4-12-82	10:00	17:00	7:00	
4-13-82	8:00	16:30	8:30	
4-14-82	10:00	17:20	7:20	
4-15-82	7:15	15:30	8:15	
4-16-82	9:38	16:20	6:42	
4-17-82				
4-18-82				Filtered clean air
4-19-82	9:03	16:05	7:02	
4-20-82	7:20	15:00	7:40	
4-21-82	8:55	16:00	7:05	
4-22-82	7:25	14:25	7:00	
4-23-82	8:30	16:00	7:30	
4-24-82				
4-25-82				Filtered clean air
4-26-82	8:30	16:40	8:10	
4-27-82	7:30	16:00	8:30	
4-28-82	7:20	14:30	7:10	
4-29-82	7:30	15:00	7:30	
4-30-82	8:30	15:15	6:45	
5-1-82				
5-2-82				Filtered clean air
5-3-82	8:54	16:15	7:21	
5-4-82	8:17	15:30	7:13	
5-5-82	7:42	16:05	8:23	
5-6-82	9:30	16:40	7:10	
5-7-82	8:30	15:30	7:00	
5-8-82				
5-9-82				Filtered clean air
5-10-82	8:40	15:50	7:10	
5-11-82	7:20	14:30	7:10	
5-12-82	7:35	15:13	7:38	
5-13-82	7:15	14:40	7:25	
5-14-82	8:22	15:27	7:05	Total NO_2 hrs. - 435
5-15-82				
				Filtered clean air
7-16-82				

Table 11

Statistical Summary of Percent Survivors

Treatment Groups	Average Survivors	REGRESSION ANALYSIS: Regression Coefficient (mortality rate)	ANALYSIS OF COVARIANCE: Homogeneity of Regression	ANALYSIS OF COVARIANCE: Analysis of Covariance
		1ST PERIOD (DAYS 28-32)		
Control	89.5	-4.47	F = 5.96*	F = 4.03*
NO_2 Exposed	80.6	-9.72	DF = 2,9	DF = 2,11
Room Control	81.1	-10.00		
		2ND AND 3RD PERIODS (DAYS 33-45)		
Control	31.0	-4.85	F = 1.20 NS	F = 6.33**
NO_2 Exposed	21.4	-4.08	DF = 2,33	DF = 2,35
Room Control	24.4	-3.75		
		4TH PERIOD (DAYS 46-50)		
Control	7.4	-2.10	F = 47.04**	F = 1.99 NS
NO_2 Exposed	5.4	0.00	DF = 2,9	DF = 2,11
Room Control	5.6	0.00		

*(Significant at 5%)

**(Significant at 1%)

NS (Not Significant)

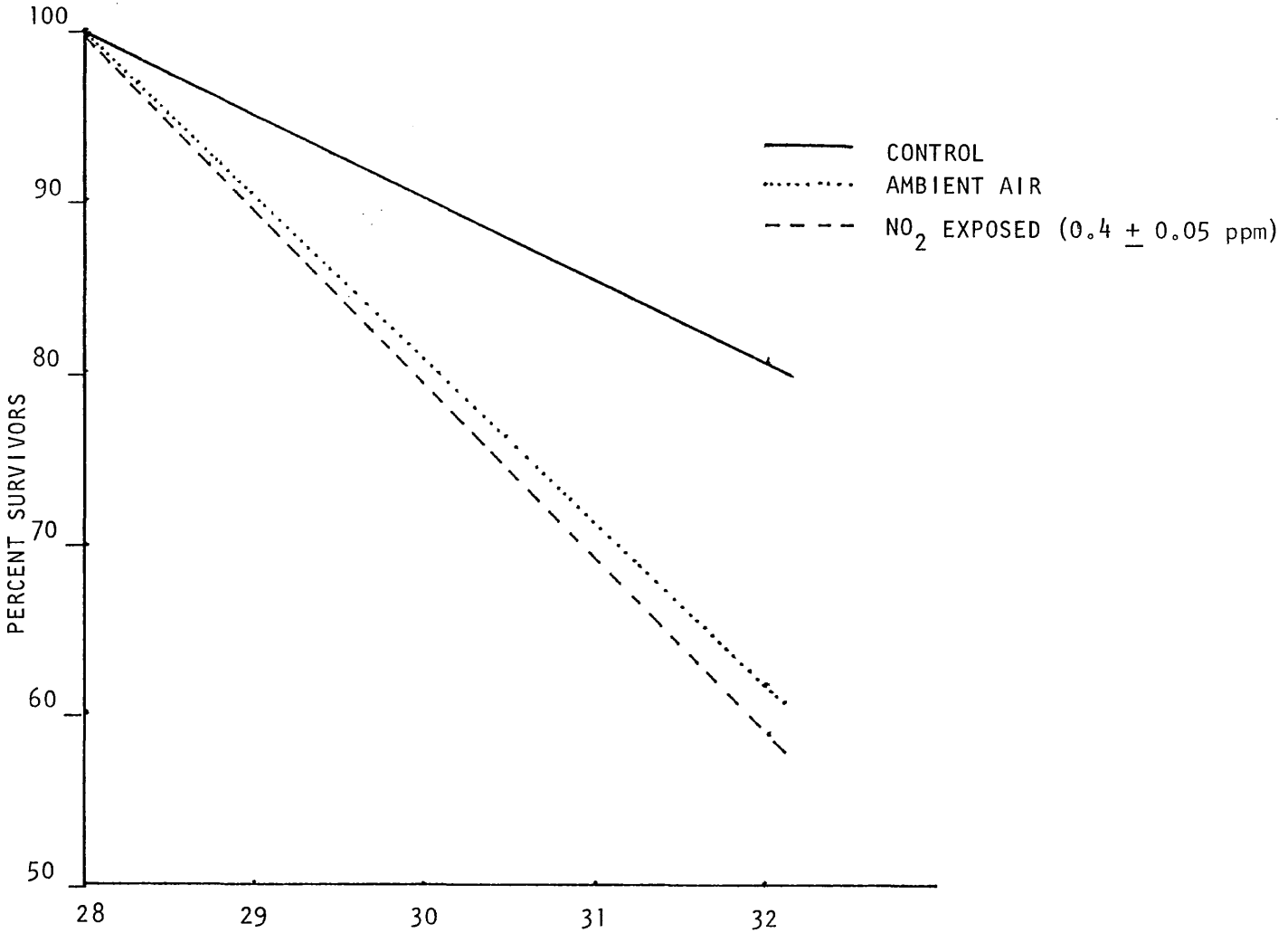

FIG. 1 DAYS AFTER MELANOMA CELL INFUSION

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10. PUBLICATIONS AND PRESENTATIONS

Utilizing the data produced by this and previous contracts we have been able to put together the following publications, which have been accepted for publication:

1) A. Richters and K. V. Kuraitis
Air Pollutants and the Facilitation of Cancer Metastasis.
Environ. Health Perspectives, in press, 1982.

2) A. Richters, V. Richters, R.P. Sherwin
Influence of Ambient Level NO_2 Exposure on Newborn and Adult Mice Body Weights.
J. Envr. Path. Toxic. and Oncology, in press, 1982.

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11. APPENDICES

Copies of the manuscripts are included as Appendices A, B, C and D.

"AIR POLLUTANTS AND THE FACILITATION OF CANCER METASTASIS"

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ABSTRACT

Studies have been carried out to determine whether the inhalation of ambient levels of nitrogen dioxide (NO_2), a common air pollutant, could influence the frequency of blood borne cancer cell metastasis to the lungs. B16 mouse melanoma cells were used as an in vivo test model. The results have indicated that animals inhaling ambient levels of NO_2 developed a significantly higher number of melanoma nodules in their lungs than the animals inhaling filtered air. Thus, a new concept for the action of air pollutants is proposed. The question is raised whether similar events are taking place in urban human populations.

The presence of pollutants in the environment, especially those with carcinogenic properties, has been of great concern to environmental health scientists. In view of this, many studies have been directed toward the identification of cancer causing agents in the environment (1-4). However, the problem of cancer involves not only the development and presence of neoplastic cells at a primary site, but also the ability of these cells to migrate, seed, and proliferate in distant organs and tissues. The importance of cancer cell dissemination and metastasis has been emphasized by many investigators and has been stated particularly well by Day, who wrote that, "even though the cause of cancer is important, in the clinical case it is the spread - the phenomenon of metastasis - that is of much more immediate concern in the human situation" (5). Moreover, considering the fact that a significant segment of the population is already affected by cancer together with the probability that one in four individuals will develop cancer (6), the question arises as to the role environmental pollutants play not only in the causation of cancer or carcinogenesis, but in the progression of the disease, particularly the dissemination of cancer cells and development of metastases.

The development and progression of cancer is a very complex process (Figure 1) and it is possible that different air pollutants could act at different sites in the sequence of cancer causation and progression. In general, one could say that certain air pollutants could participate in the process of carcinogenesis and others in the process of cancer cell dissemination and metastasis (Figure 2). With respect to carcinogenesis, noxious air pollutants could act as initiators or complete carcinogens, co-carcinogens or promoters leading to the development of cancer. In the case of cancer cell dissemination and metastasis certain air pollutants could act as facilitators by exerting their effects on the

host in a non-carcinogenic manner. Thus, individuals with existing cancer or potential cancer patients should be at highest risk since it is recognized that most cancer patients have circulating cancer cells (7,8) and in some instances cancer cells have even been demonstrated in the circulation of individuals without clinical signs of cancer (9,10). In addition, circulating cancer cells are also found in peripheral blood of tumor bearing animals (11), indicating the universality of this phenomenon. Moreover, there are several known conditions which may favor the development of cancer cell metastases from such circulating cells. The best documented of these conditions are: 1) immune suppression, 2) endothelial cell alterations, 3) cancer cell homotypic or heterotypic aggregation, 4) cancer cell interations with components of blood clotting sytem, and 5) tissue damage in general (12-17). Several of the mentioned conditions occur as a result of nitrogen dioxide (NO_2) inhalation (18-23) and thus one may expect that air pollutant inhalation could facilitate or enhance circulating cancer cell metastasis. The lung in particular is a likely candidate for such metastases development since it is a common site for metastasis in general and is affected by the inhalation of air pollutants as well. Recent experiments in our laboratory utilizing a mouse melanoma model have indeed demonstrated that inhalation of ambient levels of NO_2 facilitates blood borne cancer cell metastasis to the lungs (24-26) and the animals die if the metastatic nodules are permitted to progress. The number of melanoma nodules developing in the lungs was significantly higher ($p < 0.005$) in the exposed animals.

In this article we express a concern for human health and we present additional data from ambient level NO_2 and from ambient urban air inhalation experiments (Table 1). Most importantly, we are introducing a new concept about the possible action of air pollutants (Figure 2). More specifically, the common air pollutant (NO_2) is implicated in the facilitation of blood

borne cancer cell dissemination and lung metastases development. It should be mentioned that inhalation of cigarette smoke also has been linked to the enhancement of cancer cell metastasis by other investigators employing a different experimental system (27).

The details of our experimental methodologies have been described before (25,26). In brief, the animals (C57 BL/JC mice) were divided into three equal groups and were designated as NO_2 exposed group, filtered air control group and ambient air (vivarium room air) group. The NO_2 exposed group and the filtered air controls were housed in identical environmental chambers while the ambient air group was housed in regular vivarium room environment. The prescribed NO_2 concentration was delivered to the NO_2 environmental chamber by a method described previously (28) which has been in use in this laboratory for several years. The NO_2 levels in environmental chambers and in room air were monitored by Saltzman method (29) and a chemiluminescence NO_X detector. The animals were exposed to NO_2 7 hours/day, 5 days/week for a designated number of weeks. After the designated exposure period the three groups of animals were infused with 10^5 B16 F10R1 melanoma cells via the lateral tail vein and were housed in vivarium room air for additional three weeks. Following this time period animals were killed, lungs were removed enbloc, inflated with 10% acetate buffered formalin and the pigmented melanoma nodules were counted utilizing stereomicroscope.

We have used the foregoing experimental procedures to test the effects of 0.8, 0.5, 0.4 and 0.3 ppm of NO_2. The 0.8, 0.4, and 0.3 ppm NO_2 exposures have facilitated metastases formation in the lungs of exposed animals. The 0.5 ppm NO_2 exposure has not shown facilitation. It should be pointed out that the length of NO_2 exposure in the latter experiment was eight weeks instead of ten or 12 weeks employed in our other experiments. It is of interest that eight week exposures to ambient vivarium air also showed the lack of facilitation

(Table 1). We interpret these observations as being indicative of an exposure length response relationship, not necessarily a dose relationship. Namely, prolonged exposures to low level ambient air pollutants or low level NO_2 may be more detrimental to the host than exposures of a shorter duration to slightly higher ambient levels of pollutants.

The facilitation of metastasis development by inhalation of ambient vivarium room air is more difficult to relate to a specific air pollutant since the composition of room air could be very complex. The latter condition of course resembles closer the daily human exposures in Los Angeles or other urban areas. With respect to NO_2 levels in vivarium room air during the experimental period, the monitoring showed NO_2 levels below 0.1 ppm. In addition, experiments have been carried out in two different buildings in the same general Los Angeles area and revealed similar NO_2 levels, eliminating the possibility that a particular room or a building may play a role. The NO_2 level in the ambient outside air during the experiment period has fluctuated between 0.01 and 0.2 ppm.

The mechanism involved is not clear, but it is possible that several of the aforementioned conditions that affect the host and favor the development of cancer metastasis may be involved. Some of our own data generated from studies pertaining to the spleen may be relevant to the mechanisms involved, since we have observed different spleen responses in the early and late phases of NO_2 exposures (30,31). The latter observations considered together with other immunological studies which suggested immune stimulation in the early phases and suppression in the late phases of NO_2 exposure (18), may be part of the mechanisms involved in the facilitation of metastases development.

With respect to the human experience, epidemiological studies have shown increased mortality rates from cancer in polluted urban areas (32,33) and could be interpreted as being due to an increased frequency of metastasis.

However, other studies have not demonstrated this correlation (34,35). Most importantly, epidemiological studies designed to study specifically the frequency of metastases are missing. Several existing reports often equate the increased incidence of cancer with an increased incidence of mortality and thus present problems for appropriate interpretation. Epidemiological studies where the frequency of cancer metastases development could be correlated with a specific ambient environmental contaminant are needed urgently.

Thus, the data from our experimental studies provide the first evidence that under certain conditions inhalation of ambient levels of NO_2 or polluted urban air can facilitate blood borne cancer cell metastasis to the lungs. Even though our data comes from an experimental animal model, we consider these findings highly relevant to human health. Namely, because studies with radiation and other traumatizing treatments in cancer patients have resulted in enhancement of cancer cell metastasis and the same events have been observed with experimental animals. Thus our results with air pollutant inhalation and facilitation of blood borne cancer cell metastasis in animals may well indicate what may be happening in human populations. We feel the findings are also relevant to air quality standard setting since all available evidence should be considered.

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LEGENDS

FIGURE 1 The course of cancer. A simplified outline of cancer course. Note that metastases can also invade and enhance the progression of the destructive process.

FIGURE 2 Action of air pollutants on the process of carcinogenesis and the facilitation of cancer cell metastasis. If an air pollutant acts as facilitator it effects the host in non-carcinogenic manner.

TABLE 1 Frequency of pulmonary metastases.

FIGURE 1

THE COURSE OF CANCER

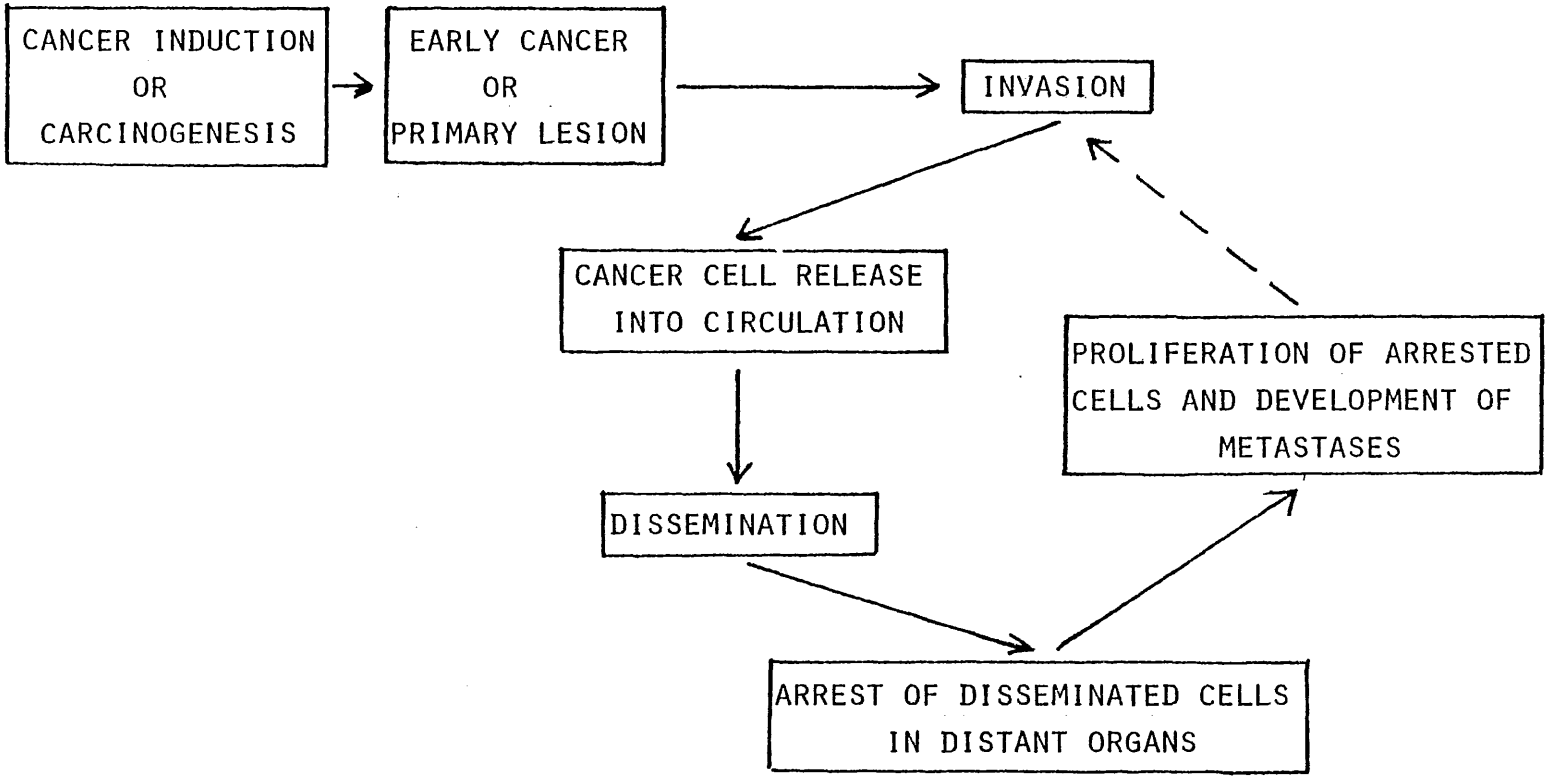

FIGURE 2

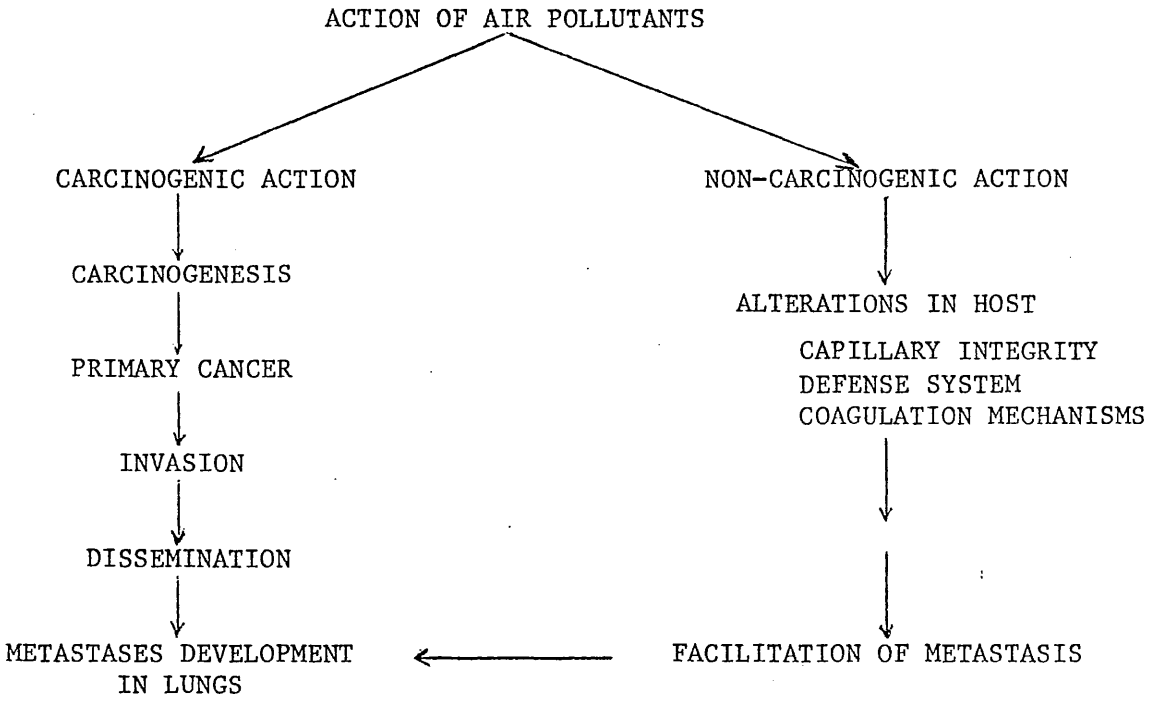
ACTION OF AIR POLLUTANTS
CARCINOGENIC ACTION
NON-CARCINOGENIC ACTION
CARCINOGENESIS
ALTERATIONS IN HOST
CAPILLARY INTEGRITY
DEFENSE SYSTEM
COAGULATION MECHANISMS
PRIMARY CANCER
INVASION
DISSEMINATION
METASTASES DEVELOPMENT IN LUNGS
FACILITATION OF METASTASIS

TABLE 1

FREQUENCY OF PULMONARY METASTASES

DURATION OF EXPOSURE	TREATMENT	NO. ANIMALS	MEAN NO. NODULES PER LUNG	P-VALUE*
8 WEEKS	FILTERED AIR	29	10.7	FA vs. NO_2 NS
	NO_2; 0.5 PPM	23	9.3	
	AMBIENT AIR	26	10.7	AA vs. NO_2 NS
12 WEEKS	FILTERED AIR	29	10.1	FA vs. NO_2 P = 0.05
	NO_2; 0.3 PPM	25	15.6	
	AMBIENT AIR	28	15.1	FA vs. AA P = 0.03
12 WEEKS	FILTERED AIR	48	35.0	FA vs. NO_2 p<0.01
	NO_2; 0.4 PPM	51	50.0	

FA = FILTERED AIR

AA = AMBIENT AIR

NS = NOT SIGNIFICANT

*MANN-WHITNEY-WILCOXON TEST

INFLUENCE OF AMBIENT LEVEL NO_2 EXPOSURE ON NEWBORN AND ADULT MICE BODY WEIGHTS

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INTRODUCTION

There are many reports of altered lung structure and function following nitrogen dioxide (NO_2) exposure (Coffin and Stokinger, 1977; Dawson and Schenker, 1979; Guidotti, 1978; Nakajima et al., 1980) and several studies have indicated extra pulmonary effects as well (Mersh et al., 1973; Sherwin and Layfield, 1974; Maigetter et al., 1978; Holt et al., 1979; Oda, et al., 1980; Miller et al., 1980). Thus, there are strong indications that NO_2 inhalation can exert systemic effects and this is not surprising since it has been shown that NO_2 or its reaction products may be distributed systemically (Goldstein et al., 1977; Yoshida et al., 1980; Parks et al., 1981). Moreover, adverse effects of NO_2 at a systemic level could be expected to influence the overall growth process, which could be reflected in the body weights of exposed animals. A simple evaluation of body weights following NO_2 exposure, especially in young developing animals, should reveal a positive or negative correlation with NO_2 inhalation exposure. To date, we are aware of only three reports focusing specifically on body weights following ambient level NO_2 exposures (Csallany and Ayaz, 1980; Kuraitis et al., 1981; Haydon et al., 1965). Of particular interest are the two studies which reported lower body weights in the NO_2 exposed animals. One of these studies (Csallany and Ayaz, 1980) combined body weight data from animals exposed to 0.50 and 1.0 ppm NO_2 and

compared the findings with respect to vitamin E supplemented and deficient diets. The results revealed that vitamin E deficiency in the control as well as the NO_2 exposed groups of mice resulted in lower body weights and that "either level of NO_2 resulted in an additional 12% decrease in body weight". The other study came from our laboratory in which we reported lower body weights in mice exposed intermittently to 0.30 ppm NO_2 for 6 weeks (Kuraitis et al., 1981). Additional NO_2 related studies mentioning body weights do exist (Kim, 1978; Kobayashi et al., 1980) but they were conducted at NO_2 levels considerably higher than those present in polluted urban air.

This report presents body weight data collected from 1590 adult and 490 newborn mice where half of the mice in each group were exposed intermittently to NO_2 at levels ranging from 0.17 to 0.80 ppm for three - twelve weeks. The histopathological observations and data from other studies utilizing the same animals are under evaluation at the present time and will be presented in separate publications.

MATERIALS AND METHODS

Source of Animals

The body weight data were collected from 13 independent experiments involving a total of 2040 mice, 450 of which were newborns and 1590 adults. The newborn mice were obtained by placing 7 day gravid S/W mice into

filtered air environments with or without added NO_2. The pups were born in the respective environmental chambers and, three weeks after the delivery were weaned, weighed, and separated into male and female groups. Only male animals were retained and exposures were continued according to particular experimental design. (Tables 1,2,3)

NO_2 Exposure

The details of NO_2 delivery system have been reported elsewhere (Sherwin and Yuen, 1972). In brief, a continuous stream of NO_2 saturated silicone fluid enters an air mixing chamber where the airflow extracts the NO_2 from silicone and delivers it to the exposure chamber. The level of NO_2 was continuously monitored with a Teco chemiluminescence NO_2 analyzer and a liquid Saltzman fluid Beckman analyzer. In addition, the NO_2 levels in the chambers were checked with a fritted bubbler (Saltzman, 1954). With one exception (experiment M108), the NO_2 exposure was intermittent, i.e. eight hours/day, five days/week for periods ranging from 1 to 12 weeks. In experiment M108 the NO_2 exposure was continuous for 3 weeks.

RESULTS

Body weight analysis of the experimental animals has been carried out according to two ranges of NO_2 exposure, i.e. from 0.50 to 0.80 ppm, and from 0.17 to 0.35 ppm.

Adult Body Weights

Table 1 presents the body weight data analysis of adult mice exposed to NO_2 levels ranging from 0.50 to 0.80 ppm.

This group includes five experiments, of which two (M127 and M113) showed significant weight differences. In M127 the exposed group of animals had a significantly lower mean body weight than the controls at the 6 and 10 week test periods ($p<.01$ and $p<.05$, respectively). At the 4 week test period, the weight difference was not statistically significant. For M113, the exposed group of animals were again lighter in weight than the control animals at the 6 week test period ($p<.05$), but at 12 weeks the exposed animals actually had a greater mean body weight.

Table 2 presents the body weight analysis of adult mice exposed to NO_2 levels ranging from 0.17 to 0.35 ppm. There was a total of five experiments with NO_2 exposures from 1 to 12 weeks. Two significant differences were found, i.e. a higher weight for the exposed group of animals ($p<.025$) at 7 weeks (M136) as well as at 2 weeks ($p<.05$; M126).

Newborn Body Weights

There were three experiments (Table 3) where newborn mice were exposed to NO_2 levels ranging from 0.25 to 0.30 ppm for 3 to 12 weeks. Of the six test periods, four showed lower body weights for the exposed animals at statistically significant levels (from $p = 0.05$ to $p<.005$) and the others had similar trends.

DISCUSSION AND CONCLUSIONS

The experiments described in this report have utilized large numbers of animals and have compared body weights of adult and newborn animals after the inhalation of NO_2 at ambient levels for different time periods. In the experiments utilizing adult mice, the results, with few exceptions, show a lack of NO_2 influence on the body weights (Tables 1,2). The few instances with decreased or increased weights in NO_2 exposed groups may reflect animal groups with different sensitivities but a definitive trend can not be recognized. Another possibility for the changes in body weights is the change in feeding habits, which was not monitored in these studies but in studies by other investigators where animals have been exposed to high level NO_2, changes in feeding habits have been observed (Kobayashi et al., 1980).

In contrast, the findings in newborn animal experiments consistently showed lower mean body weights in the NO_2 exposed groups. Moreover, in most of the experiments the difference was statistically significant (Table 3). These lower body weights are strongly suggestive of a slowing in the developmental growth process. Thus, it is concluded that the newborn mice are much more sensitive to the inhalation of ambient levels of NO_2 and that the adverse effect is partially reflected by a significantly smaller body weight gain. This conclusion is further sup-

ported by the significantly lower mean body weights observed in experiment M127 (Table 1) in which case the adult mice were the youngest at the onset of the exposure regimen. However, the mechanisms responsible for the observed body weight changes have not been identified, and it is quite possible that more than one mechanism is involved. At this time one can only speculate that alterations in neurologic responses and/or changes in the metabolic activity as well as structural and functional changes of vital organs may play an important role in affecting body weight gains.

While this is the first report demonstrating significant differences in mean body weights of newborn mice exposed to ambient levels of NO_2, it should be mentioned that similar observations have been reported after utilizing high levels of NO_2 (10 ppm) on newborn rats (Freeman et al., 1974). It is important to re-emphasize that the results presented in this report dealt with the inhalation of ambient levels of NO_2. Therefore, we believe that newborn body weight comparisons, among exposed and control animals may provide additional simple information pertinent for a more precise assessment of the risks associated with ambient level inhalation of NO_2. It still remains to be determined if the body weight discrepancies observed will remain permanent throughout the life of the animals and whether correlations can be made with histopathological or functional body changes. Most

importantly, the findings with newborn animals make one ask the question if similar events may be taking place in human populations living in polluted urban environment and we should strive to obtain the answers.

Acknowledgments

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TABLE 1

BODY WEIGHTS OF ADULT NO_2 EXPOSED AND CONTROL S/W MICE

Experiment Number	NO_2 Exposure Level (ppm)	NO_2 Exposure Time (weeks)	Control			Exposed			P Value
			$\bar{M}$	(gms)	SD	$\bar{M}$	(gms)	SD	
M108	0.50 ± 0.05	3	31.31	(36)*	2.76	32.36	(36)*	3.80	NS
M109	0.80 ± 0.10	3	33.43	(24)	3.69	34.03	(24)	4.22	NS
		6	34.27	(23)	4.56	34.05	(22)	5.23	NS
M111	0.60 ± 0.05	3	35.69	(36)	2.17	36.28	(36)	2.59	NS
		6	38.39	(36)	3.13	39.36	(36)	3.34	NS
		12	41.67	(26)	3.23	42.05	(28)	2.80	NS
M113	0.80 ± 0.05	6	40.50	(26)	3.10	38.80	(25)	3.60	<0.05
		12	40.95	(42)	2.90	43.50	(44)	3.50	→0
M127	0.75 ± 0.05	4	28.66	(10)	2.58	29.22	(10)	3.37	NS
		6	32.64	(20)	2.64	30.58	(20)	2.73	<0.01
		10	35.47	(20)	2.68	33.98	(20)	2.83	<0.05

*( ) indicates number of animals studied

S/W - Swiss Webster

P - Student's t-test

$\bar{M}$ - Mean

SD - Standard Deviation

TABLE 2

BODY WEIGHTS OF ADULT NO_2 EXPOSED AND CONTROL S/W MICE

Experiment Number	NO_2 Exposure Level (ppm)	NO_2 Exposure Length (weeks)	Control			Exposed			P Value
			$\bar{M}$	(gms)	SD	$\bar{M}$	(gms)	SD	
M114	0.34 ± 0.02	6	36.94	(60)*	2.91	36.65	(60)*	2.83	NS
M123	0.30 ± 0.03	6	32.60	(150)	2.46	32.53	(150)	2.64	NS
M126	0.17 ± 0.04	1	30.82	(10)	4.54	28.50	(10)	2.10	NS
		2	31.07	(20)	2.68	32.73	(20)	3.16	<0.05
		3	31.99	(10)	1.03	31.80	(10)	3.67	NS
		4	32.86	(20)	1.84	33.39	(20)	2.66	NS
		5	32.37	(20)	3.27	33.11	(20)	2.85	NS
		6	34.69	(20)	2.17	33.94	(20)	2.39	NS
M135	0.30 ± 0.05	12	35.27	(25)	3.24	35.58	(25)	4.62	NS
M136	0.30 ± 0.05	6	37.19	(10)	2.86	36.14	(10)	2.83	NS
		7	31.64	(10)	2.11	33.96	(10)	2.46	<0.025
		8	32.04	(10)	4.01	33.45	(10)	3.81	NS
		9	36.15	(10)	2.44	35.02	(10)	3.20	NS
		10	35.02	(10)	3.20	35.01	(10)	2.81	NS

*( ) indicates number of animals

S/W - Swiss Webster

P - Student's t-test

$\bar{M}$ - Mean

SD - Standard Deviation

TABLE 3

BODY WEIGHTS OF NO_2 EXPOSED AND CONTROL NEWBORN S/W MICE

Experiment Number	NO_2 Exposure Level (ppm)	NO_2 Exposure Length (weeks)	Control			Exposed			P Value
			$\bar{M}$	(gms)	SD	$\bar{M}$	(gms)	SD	
M116	0.25 ± 0.05	3	9.19	(81)*	2.14	8.27	(60)*	2.10	<0.005
		12	34.95	(37)	1.94	33.31	(41)	3.49	<0.01
M117	0.25 ± 0.05	3	10.51	(55)	2.63	9.85	(77)	2.48	=0.05
		6	25.22	(15)	3.82	24.36	(15)	3.85	NS
M119	0.30 ± 0.05	3	10.03	(81)	2.42	9.81	(75)	2.70	NS
		6	25.13	(52)	3.54	23.42	(52)	4.62	<0.025

*( ) indicates number of animals studied

S/W - Swiss Webster

$\bar{M}$ - Mean

SD - Standard Deviation

P - Student's t-test

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A New Relationship Between Air Pollutant Inhalation and Cancer

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Abstract

Many studies have been conducted to investigate the effects of different air pollutants on health. Our studies have focused on the effects of nitrogen dioxide (NO_2), and recently we have reported that inhalation of low levels of NO_2 can facilitate cancer cell metastasis. The study described in this report utilized the same B16 mouse melanoma metastasis model of previous investigations, but under different NO_2 exposure conditions. The results provide further evidence that inhalation of ambient level NO_2 (0.4 ppm) or polluted urban ambient air play a role in facilitation of blood borne cancer cell metastasis. In addition, results show different pattern of melanoma cell distribution in the lungs of NO_2 and ambient air exposed animals. They also indicate that extended periods of clean air between NO_2 exposures may diminish the severity of the insult in the less sensitive animals. It is our conclusion that the results provide strong support for the need of improved air quality and for reduction of noxious pollutants in urban ambient air.

Introduction

One of the common air pollutants in many urban environments is nitrogen dioxide (NO_2) and numerous studies have been carried out to determine the health hazards associated with inhalation of NO_2. In general, the studies have indicated that under certain conditions animals inhaling NO_2 develop structural and functional changes in the lungs, alterations in the body's defense system, and abnormal biochemical, physiological and enzymatic reactions (1-6). In addition, the respiratory function impairment has been observed in residents of polluted areas (7). The severity and the type of response, however, depends on the concentration and the length of NO_2 exposure. Further, the role of air pollutants in carcinogenesis and mutagenesis also has been intensively investigated, and several air pollutants have been identified as carcinogens and/or mutagens (8-11). Although NO_2 has not been classified as such (12), some epidemiological studies have suggested that there is a relationship that exists between the increased incidence of cancer and ambient concentration of nitrogen oxides (13,14). In addition, the in vivo formation of nitrosamines (potential carcinogen) under NO_2 influence has been demonstrated in some studies (15), but other investigations have failed to detect such reactions (16).

Our studies have focused on investigating NO_2 relationship to the spread of cancer or metastasis, an area that has been neglected, and have shown that inhalation of NO_2 at an ambient level plays a role in facilitation of blood borne cancer cell

metastasis to the lungs (17,18). Thus, we have been able to demonstrate a new relationship between a common air pollutant (NO_2) and cancer, i.e. facilitation of metastasis. The recognition of this NO_2 inhalation effect in an experimental system raises the question that similar events may take place in human population. Our results also suggest that other air pollutants in ambient air, alone or in combination, may play a similar role in cancer metastasis. The facilitation should not be confused with "positive modifiers" (19) which modulate some step in carcinogenesis. Facilitators, on the other hand, influence the already existing cancer cell dissemination by affecting the host without participation in carcinogenesis (20). Since cancer metastasis is one of the most significant events in cancer progression, it becomes important to identify the specific air pollutants and the conditions under which facilitation of cancer metastasis may occur. In this report, we are presenting experimental data from a study which investigated and compared the effects of a specific NO_2 exposure condition and Vivarium ambient air. Results suggest that the distribution and the development of lung metastases from the blood borne cancer cells may be influenced by the frequency of ambient level NO_2 exposure. Further, the study demonstrates different susceptibility to NO_2 and polluted urban ambient air among experimental animals. The latter observation is not surprising since population subsets with different sensitivities are well recognized among humans (21,22).

Materials and Methods

The details of our experimental methodologies have been described before (18). In brief, 5-week old C57Bl/6J male mice were divided into three experimental groups: 1) NO_2 exposed; 2) filtered, NO_x and O_3 free, clean air control, and 3) ambient room air (Vivarium room air). The NO_2 group had 31 animals (1 extra animal for possible attrition), while the other two groups had 30 animals each. The NO_2 exposed and the filtered air controls were housed in identical environmental chambers while the ambient air group was housed in regular Vivarium room environment. The predetermined NO_2 concentration (0.4 ± 0.05 ppm) was delivered to the NO_2 experimental chamber by a method which has been described before (23) and has been used in this laboratory for several years. The NO_2 levels in environmental chambers and in room air were monitored by Saltzman's method (24) and a chemiluminescence NO_x detector. The room air was also monitored for O_3. Animals were exposed to NO_2 7 hours a day, 5 days per week, with intermittent extended clean air periods between NO_2 exposure, for a total NO_2 exposure of 420 hours. The 420 hours of intermittent exposure was carried out as follows: 1 week of clean air exposure followed the initial 6 week NO_2 exposure and each of the two 3 week exposures. After the third clean air exposure, the animals were infused intravenously with 8.7×10^4 Bl6 Fl0 Rl melanoma cells. One animal in control group was lost during cell infusion procedure. Three weeks later the animals were killed, their lungs removed en bloc and fixed by perfusing the bronchial tree with acetate buffered formalin. After fixation the lobes of each lung were

separated. Melanoma nodules were counted utilizing Leitz stereomicroscope with 4X objective and 12.5X occular. The nodules were superficial enough to permit an accurate evaluation. Sectioning of the lungs has revealed only occasional melanoma nodules in central lung parenchyma at this stage of metastasis and counting of nodules on gross specimens has proved to be a very satisfactory method. The data were evaluated using the Mann-Whitney statistical test. In addition, upper quartile analysis was carried out on the data derived from the area of the distribution curve associated with upper quartile.

Results

The data is summarized in Table 1. The average number of melanoma nodules was tabulated for each lobe of the lung and was the greatest in the lungs of animals inhaling ambient Vivarium room air. With respect to melanoma nodule distribution in different lobes of the lungs, the average number of nodules was the greatest in the left lung and the right lower lobes for all three experimental groups. The statistical analysis of the number of nodules per lung is presented in Table 2. The number of melanoma nodules was significantly higher ($p = 0.008$) in the animals housed in ambient room air than in the filtered clean air group. The NO_2 exposed group showed higher number of nodules than the controls but the difference was not significant ($p = 0.0747$). Analysis of the upper quartile data showed that both the NO_2 and the ambient room air groups have significantly higher numbers of melanoma nodules than the controls (Table 3), indicating that we are dealing with animals showing different susceptibilities not only to NO_2 but other pollutants as well. In addition, the number of melanoma nodules in different lobes of the lungs from each experimental group were also compared and the statistical analysis is presented in Table 4. It is of interest that in NO_2 exposed group there were significantly more melanoma nodules only in right upper and right lower lobes, while the ambient air group showed significantly higher melanoma numbers in all right lobes. There were no significant differences noted between the left lungs and the cardiac lobes among the three groups. However, using the upper quartile data and comparing

left lung vs. right lung, the NO_2 exposed as well as the ambient room air group animals showed significantly greater number of melanoma nodules than controls. It is of interest that by comparing only the right lower lobes among the different groups, significant differences can be detected (Table 5). The latter observations suggest that the left lung and the right lower lobe may be the most vulnerable portions of the lungs as revealed by the upper quartile analysis. No significant differences were noted between NO_2 and ambient air groups.

Discussion

The results from our previous studies have indicated that intermittent NO_2 exposures at levels of 0.3 ppm - 0.8 ppm for a period of 350 hours or longer will induce metastasis facilitation in exposed animals at a significantly higher incidence than in controls. In the present study, extended clean air periods were introduced between NO_2 exposure episodes to see if this would alter the previously observed incidence of lung metastases development. The rationale for changing the exposure schedule was based on the assumption that if incidence of metastasis to the lungs decreased, the extended clean air episodes between NO_2 exposures either reduced the severity of the insult or provided some other benefit to the less sensitive segment of the test animals. The results indicate that even with extended clean air periods, the NO_2 exposed animals developed more melanoma nodules than the controls but the difference is not significant ($p = 0.075$). In contrast, the animals breathing ambient urban air (Vivarium room air), develop significantly

greater number of melanoma nodules in their lungs than the control group ($p = 0.008$), Table 2. However, the comparison of upper quartile data of the three groups shows that the animals of both exposure groups (X and R) have a significantly greater number of melanoma nodules in their lungs (Table 3). This, of course, strongly suggests that under these exposure conditions, the animals of the same group exhibit different susceptibilities and the more sensitive animals are detected by the upper quartile analysis. The latter point is reinforced in Table 5 where left and right lungs are compared. The results clearly show highly sensitive populations in both NO_2 and room air exposed groups develop significantly greater numbers of melanoma nodules in their lungs than the controls. It is tempting to postulate that extended clean air periods between ambient level (0.4 ppm) NO_2 exposures, may permit partial recovery or at least reduce the severity of NO_2 effects in the less sensitive animals. The latter, if confirmed, would be an extremely important finding.

With respect to Vivarium room air it should be pointed out that the occasional monitoring of NO_2 and O_3 in the room air revealed NO_2 levels varying from 0 - 0.12 ppm and O_3 levels between 0 - 0.07 ppm. But on the basis of the outside ambient air studies, it is very likely that the room air at times contained higher concentrations of NO_2 and O_3 as well as other pollutants. The complexity of ambient air cannot be overemphasized and may account for our results. Most importantly,

similar air is encountered daily in urban communities. Table 6 gives the concentrations of some of the ambient air pollutants in the vicinity of the building where our experiments were carried out. This information was compiled from data provided by the California Southcoast Air Quality Management District and shows that December 1981 was the worst month for this location in terms of several pollutants. Even though the table does not provide a complete list of existing air pollutants, it is obvious that during the period of our experiment, we were dealing with a mixture of diverse pollutants in the ambient air. Moreover, several of these pollutants showed several days of high levels as indicated by the daily maxima and the range in Table 6. On the basis of our results and the foregoing information, we suggest that the observed greater effect of ambient air may be attributable to the mixture of pollutants or to one of the pollutants other than NO_2. In addition, it appears that various pollutants may affect the individual lobes of the lungs differently, Table 4. While the NO_2 exposure has not significantly influenced cancer cell metastasis to the right middle lobe, breathing of the ambient air has produced significant differences in all three right lobes, probably reflecting the complexity and the different mode of action of the ambient air pollutant mixture.

With respect to cancer cell metastasis, it should be noted that the blood borne cancer cell metastasis represents only a small part of the complex process of cancer progression, and was used in these studies as a probe to detect adverse NO_2

inhalation effects. A simplified outline of cancer progression is presented in Figure 1. It can be seen that cancer invasion and/or infiltration can progress via surrounding tissues, blood vessels or lymphatics. These routes can be involved singly or in combination and they are interconnected. The latter makes cancer progression, dissemination and metastasis a continuous propogating process, unless intervened by successful therapy.

On the basis of what is known about common ambient level air pollutant inhalation effects (1-6), we can indicate and postulate several sites at which an air pollutant action could facilitate the course of cancer progression. The most likely steps to be affected by air pollutants are indicated by arrow heads. In order to demonstrate the specific mechanisms responsible for the facilitating action, several possibilities will have to be considered as we have indicated in an earlier study (18). Moreover, one should bear in mind that cancer progression depends not only upon conditions of the host, but also upon the properties of the cancer cells. The interaction between the host and the cancer cell properties determines the final outcome. The major host functions and the tissue structures, as well as cancer cell properties, which are often associated with cancer growth and progression, are listed in Table 7.

It is pertinent to emphasize that the findings of our studies even in an experimental animal system, warrant concern about inhalation of noxious air pollutants in general and its effects on human urban populations in particular. Such concern is particularly important in view of the estimation that 1 out of 4 individuals will develop cancer during their lifetime (25)

and that many cancer patients have circulating cancer cells in their blood (26,27). Some epidemiological studies correlating increased cancer mortality with polluted urban environment may be indicative of such events (28,29). However, specific epidemiological studies where the time and frequency of metastases occurrence have been correlated with air pollutant exposure are missing. Most importantly, the data discussed in this paper provide additional support for the need of improved air quality and the reduction of ambient air pollutants. Further studies evaluating other air pollutants and exposure condition effects on cancer metastasis are needed. Most importantly, such information should assist regulatory agencies in making decisions on air quality standards.

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Legend Figure 1.

Cancer progression. The main routes of cancer invasion and dissemination are indicated by heavy arrow lines and the interconnections by thinner arrows. Many cancer cells will die along the routes of progression but the surviving cells will develop into metastases which in turn will invade and metastasize.

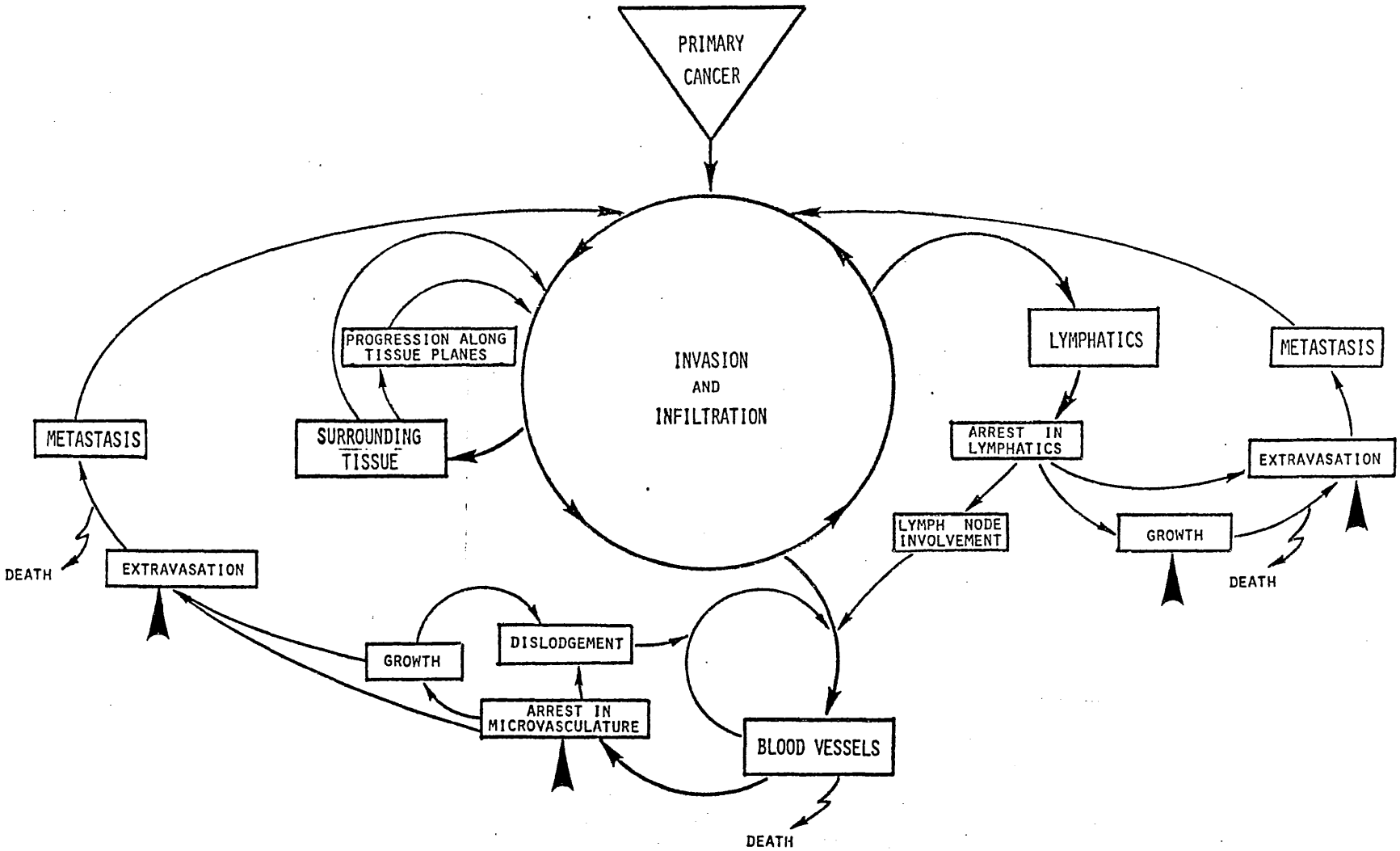

FIG. 1 PROGRESSION OF CANCER

Table 1

Melanoma Nodule Distribution in the Lungs*

Group	Treatment	No. Animals	Left† Lobe	Right Upper Lobe	Right Middle Lobe	Right Lower Lobe	Cardiac Lobe	No. Nodules Per Lung
C	Filtered clean air	29	4.90 (0-10)	4.34 (0-15)	2.93 (0-11)	4.38 (0-11)	1.86 (0-4)	18.41 (0-38)
X	0.4 ± 0.05 ppm $\bar{N}O_2$	31	7.71 (0-22)	6.80 (0-20)	3.81 (0-11)	7.81 (0-23)	2.45 (0-9)	28.58 (0-89)
R	Ambient room (Vivarium) air	30	7.83 (1-23)	6.03 (0-17)	5.73 (0-18)	8.27 (0-23)	2.87 (0-7)	30.73 (4-66)

( ) - range

* - mean number of nodules

† - left lobe = left lung

Table 2

Incidence of Melanoma Nodules Per Lung

Group	Treatment	No. Animals	$\bar{M}$	S.D.	MW-Test Probability
C	Filtered clean air	29	18.41	11.94	C vs. X 0.0747
X	0.4 ± 0.05 ppm $\bar{N}O_2$	31	28.58	22.25	
R	Ambient room (Vivarium) air	30	30.73	19.00	C vs. R 0.0080

$\bar{M}$ - mean number of melanoma nodules per lung

SD - standard deviation

MW - Mann Whitney

Table 3

Incidence of Melanoma Nodules Per Lung

Upper Quartile Analysis

Group	Treatment	$\bar{M}$	S.D.	MW-Test Probability
C	Filtered clean air	36.60	8.26	C vs. X 0.0137
X	0.4 $\pm$ 0.05 ppm $\overline{NO}_2$	63.50	17.11	
R	Ambient room (Vivarium) air	65.60	11.50	C vs. R 0.0122

$\bar{M}$ - mean number of nodules

SD - standard deviation

MW - Mann Whitney

Table 4

Comparison of Melanoma Nodule Numbers

in Different Lobes of the Lungs

Treatment Groups	Mann-Whitney Test Probabilities				
	Left† Lobe	Right Upper Lobe	Right Middle Lobe	Right Lower Lobe	Cardiac Lobe
Controls vs. 0.4 ± 0.05 ppm $\bar{N}O_2$	0.1905^{NS}	0.0026^{*}	0.1169^{NS}	0.0458^{*}	0.6787^{NS}
Controls vs. Ambient room (Vivarium) air	0.0909^{NS}	0.0458^{**}	0.0017^{**}	0.0026^{**}	0.1677^{NS}

NS - not significant

* - significantly higher in NO_2 exposed.

** - significantly higher in ambient room air group.

† - left lobe = left lung

Table 5

Upper Quartile Analysis of Melanoma

Nodules in Left and Right Lungs

Treatment Groups	Mann-Whitney Probabilities		
	Left Lung	Right Lung	Right Lower Lobe
Control vs. 0.04 + 0.05 ppm NO_2	0.0024*	0.0066*	0.0006*
Control vs. Ambient room (Vivarium) air	0.0009**	0.0082**	0.0010**

* - significantly higher in NO_2 exposed

** - significantly higher in ambient room air group

Table 6

AVERAGE DAILY 1 HR MAXIMA OF AIR POLLUTANTS

August - December 1981

Pollutant	August	September	October	November	December
CO[1]	4.0 (2.0-10.0)	4.0 (1.0-9.0)	6.0 (2.0-12.0)	7.0 (2.0-12.0)	9.0 (2.0-18.0)
SO_2[1]	0.02 (0.00-0.03)	0.02 (0.00-0.05)	0.02 (0.00-0.03)	0.02 (0.00-0.04)	0.03 (0.00-0.04)
NO_2[1]	0.13 (0.06-0.42)	0.14 (0.06-0.30)	0.09 (0.04-0.23)	0.13 (0.04-0.29)	0.19 (0.04-0.45)
NO[1]	0.15 (0.02-0.44)	0.17 (0.01-0.50)	0.26 (0.04-0.65)	0.45 (0.09-0.82)	0.54 (0.11-0.89)
NO_x[1] $NO^x + NO_2$	0.23 (0.08-0.58)	0.26 (0.06-0.65)	0.33 (0.07-0.73)	0.54 (0.15-0.96)	0.67 (0.19-1.05)
O_3[1]	0.15 (0.04-0.27)	0.12 (0.04-0.22)	0.07 (0.03-0.14)	0.05 (0.01-0.10)	0.03 (0.00-0.08)
Hydrocarbons[1] as methane	49.0 (26.0-101.0)	48.0 (22.0-90.0)	48.0 (23.0-85.0)	65.0 (33.0-106.0)	69.0 (30.0-135.0)
Methane[1]	46.0 (25.0-101.0)	45.0 (20.0-90.0)	45.0 (22.0-77.0)	58.0 (32.0-80.0)	60.0 (27.0-117.0)
Nitrate[2]	21.3 (16.6-21.3	23.9 (13.9-23.9)	26.6 (7.6-26.6)	21.7 (9.7-21.7)	49.6 (5.9-49.6)
Sulfate[2]	13.5 (7.5-13.5)	20.9 (12.2-20.9)	16.4 (5.4-16.4)	12.4 (7.5-12.4)	15.3 (4.6-15.3)
Lead[2]	1.13 (0.64-1.13)	0.86 (0.39-0.86)	2.12 (0.54-2.12)	1.52 (0.62-1.52)	2.37 (0.43-2.37)
Total susp.[2] particulates	143.0 (96.0-143.0)	150.0 (78.0-150.0)	149.0 (80.0-149.0)	122.0 (74.0-122.0)	219.0 (49.0-219.0)

[1]Parts per million = ppm

[2]$\mu g/m^3$ - Max. 24 hr average

( ) = range

Table 7

Factors Associated with Blood Borne Cancer Cell Dissemination and Metastases Development

Host Factors	Cancer Cell Properties
Defense system	Viability
Vascular integrity	Mobility
Hormonal activity	Cell surface
Hemodynamics	Antigenicity
Hemostasis	Enzymatic activity
Thrombosis	Metabolic activity
Coagulative factors	Mitotic activity
	Thromoboplastic activity
	Fibrinolytic activity

APPENDIX D

DO NOT FOLD

13th International Cancer Congress
Official Abstract Form

FOR OFFICE USE ONLY	
Temp. Abst. No.	Perm. Abst. No.

CATEGORIES AND SUBCLASSIFICATIONS
(Fill in ■ one category and one subclassification only)

BP		DR		PT		CC		CG	
01		01		01		01		01	
02		02		02		02		02	
03		03		03		03		03	
04		04		04		04		04	
05		05		05		05		05	
06				06		06		06	
07		EP		07		07		07	
08		01		08		08		08	
09		02		09		09		09	
10		03		10		10		10	
11		04		11		11		11	
12		05		12		12			
13		06		13		13		CMT	
		07		14					
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02		01		RB		02		DM	
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DEADLINE FOR RECEIPT OF ABSTRACTS IS DECEMBER 1, 1981

FACILITATION OF CANCER CELL METASTASIS BY INHALATION OF AIR POLLUTANTS. Arnis Richters, Valda Richters, and Russell P. Sherwin, Department of Pathology, University of Southern California, Los Angeles, CA 90033, U.S.A.

Experiments were designed to determine if inhalation of ambient level nitrogen dioxide (NO_2), a common air pollutant, can influence the incidence of metastases development in the lungs. Groups of C57Bl/6J mice were exposed to 0.3, 0.4, 0.5 or 0.8 parts per million of NO_2 for different time periods. The control groups inhaled filtered NO_2 free air or ambient room air. Following the exposure the animals were infused intravenously with 10^5 B16 mouse melanoma cells and three weeks later the number of melanoma nodules in the lungs were quantitated. The results indicate that under certain exposure conditions the animals exposed to ambient level NO_2 developed a significantly higher number ($p<0.005$) of melanoma nodules in their lungs than the animals inhaling filtered NO_2 free air. Similar results were obtained with animals inhaling ambient air. The data indicate that under certain conditions the inhalation of ambient level NO_2 or ambient air may alter the host in a manner which facilitates the blood borne cancer cell metastasis to the lungs. The specific mechanisms responsible for this have not been identified at this time. In view of the findings the question is raised if similar events are taking place in human urban population. In addition the data may be of assistance in making decisions on air quality standards. (Supported by contracts No. A9-076-31, A0-106-32 from the State of California Air Resources Board).

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