FINAL REPORT

ARB Contract A2-128-33

The role of air pollutants in facilitation of cancer cell metastasis

Period: June 8, 1983 - December 31, 1984

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1. ABSTRACT

In recent years we have established an animal model (mouse) in which we have utilized a biological probe - circulating cancer cells - to detect adverse effects following NO_2 inhalation. The indicator for the adverse NO_2 effects has been the increased incidence of lung metastases in the exposed animals. With respect to the latter, our studies have indicated that inhalation of NO_2 facilitates blood borne cancer cell metastasis to the lungs of exposed animals. This is a new aspect of NO_2 inhalation hazards which has not been recognized before our studies. The studies described in this report were designed to improve the sensitivity of this method and to employ it to evaluate other air pollutant effects. The experiments were also carried out to determine the effects of NO_2 on the growth of a primary rat carcinoma. With respect to the latter, adenocarcinoma cells were transplanted into the mammary fat pads of the exposed and control rats and this transplant was considered to be a "primary" cancer. In all of the experiments there were two groups of animals; one was exposed to an ambient concentration of a particular air pollutant and the other received clean filtered air. The animals were exposed in special environmental chambers, with continuous monitoring of the NO_2 or O_3 concentrations. Following the appropriate exposure periods, all of the mice were injected intravenously with cancer cells (biological probe). The functioning of mouse splenic natural killer cells was also evaluated following NO_2 exposure. After three weeks the lungs were examined for the presence of melanoma nodules or metastases. The results have indicated that by utilizing a lower number of cells in the biological probe, the sensitivity of the method could be improved. We have been able to demonstrate that the mice exposed to 0.4 ± 0.05 ppm of NO_2 developed not only significantly more metastases in their lungs than the controls, but that there were significantly more mice with metastases in the exposed group. The NO_2 apparently also enhanced the growth of the transplanted cancer cells in those animals which were more sensitive to the NO_2 insult. The animals exposed to 0.15 ± 0.02 ppm of O_3 also developed more metastases in their lungs than the controls, but the difference was not statistically significant and the meaning of the this finding was not clear. The results also revealed significantly lower body weights in O_3 exposed animals which indicated that even at this low level

the animals were affected adversely by O_3. The experiment with spleen cells suggested that the exposed and the control animal spleen cells responded differently to the cancer cells, reflecting some ozone affect on the spleen cells. In general, these experiments have provided new information which indicates that inhalation of common air pollutants at ambient levels may be more hazardous than previously recognized. Even though these studies were carried out in animal model systems, we have to suspect that the same or similar events may take place in humans residing in a polluted environment. This may be particularly true with individuals who are sensitive to air pollutants and those who have various diseases, including cancer.

2. ACKNOWLEDGEMENTS

Principal investigator wishes to acknowledge the assistance of the following personnel: Russell P. Sherwin, M.D., Valda Richters, Ph.D., Dolores Oliver, Walter Wiegand and the ARB El Monte personnel.

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."

4. TABLE OF CONTENTS

	page
Abstract	1-2
Acknowlegements	3
Disclaimer	4
Table of Contents	5
List of Tables	6
Summary and Conclusions	7-8
Recommendations	9-10
Body of Report	11-19
Design, Materials, and Methodology	12-15
Results	15-17
Discussion	17-20
References	23-24
Publications and Presentations	25
Appendices	26

5. LIST OF TABLES

Table 1. Incidence of melanoma nodules in lungs and among animals, 0.4 ± 0.05 ppm NO_2 exposure. 21

Table 2. Growth and metastasis of rat mammary adenocarcinoma, days postimplantation, 0.4 ± 0.05 ppm NO_2 exposure. 21

Table 3. Growth and metastasis of rat mammary adenocarcinoma days postimplantation, 0.4 ± 0.05 ppm NO_2 exposure. 21

Table 4. Incidence of lung metastases in animals exposed to 0.15 ± 0.02 ppm O_3. 22

Table 5. Spleen cell effects on metastatic potential of blood borne melanoma cells. 22

6. SUMMARY AND CONCLUSIONS

The experiments under this contract were used to investigate the following: first, an experiment was designed to test not only the incidence of lung metastases development from blood borne cancer cells in NO_2 exposed animals, but also the incidence of metastasis among exposed animals; second, to determine if O_3 inhalation at 0.15 ppm facilitates blood borne cancer cell metastases development in the lungs; third, to study NO_2 effects on the growth and spread of simulated primary cancer; and fourth, to carry out preliminary studies to identify the mechanisms involved in facilitation of metastasis, particularly with respect to the immune system. Our experimental approach was similar to our earlier studies that is, we utilized a biological probe - blood borne cancer cells - to detect the adverse inhalation effects. A new model utilizing a transplantable rat mammary adenocarcinoma was also tested to simulate primary cancer growth and progression. In both of these models the development of increased incidence of lung metastases in exposed animals would indicate that an adverse effect has taken place. This type of approach permits us not only to obtain indicators for adverse effects, but it shows how pollutant inhalation could effect the progression and the spread of cancer. The latter has great relevance to human disease. With respect to blood borne cancer cells we have clearly shown that both the incidence of lung metastases and the incidence of metastasis among animals is significantly increased in exposed animals. With O_3 the answer is not as clear at the present time since we had an increased incidence of metastases in exposed animals, but it was not statistically significant. However, there was a significant decrease in body weight ($P < 0.005$) in O_3 exposed animals, which implies adverse effect. The results from the transplantable mammary adenocarcinoma are not conclusive at this time, but there is suggestion that NO_2 may facilitate cancer growth in more sensitive animals. The preliminary experiments to detect impairment in the immune system, particularly the natural killer cells, are suggestive of adverse NO_2 effects on spleen cells, however, more experiments are needed to reach specific conclusions.

Finally, the findings of these experiments provide new data that inhalation of certain ambient concentrations of NO_2 or O_3 facilitate blood

borne cancer cell metastasis to the lungs or induce other abnormalities.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 these and other findings lend strong support for being concerned about urban air quality. The mechanisms involved in facilitation of metastasis still remain unknown but the clues obtained should justify further studies. Since the major concern is the air quality and how pollutant inhalation may effect human health, the data obtained from our model system should receive consideration in making decisions on air quality standards.

7. RECOMMENDATIONS

The basic unit of our body is the living cell and alterations in function or structure of these cells are usually detrimental to our health. The cellular functions and responses are influenced by a variety of environmental factors. Our studies have been focused on the effects of common air pollutants, such as nitrogen dioxide and ozone. The studies have demonstrated that inhalation of nitrogen dioxide at levels which are frequently encountered in Los Angeles ambient air play a role in the facilitation of blood borne cancer cell spread or metastasis to the lungs of exposed animals. The effect is even more dramatic in animals which are more sensitive to the insult of NO_2, as indicated by their early death from lung metastases. Similarly, the same exposure level also appears to enhance the growth of cancer cells transplanted into the mammary fat pads, but only in a fraction of animals, which again suggests increased sensitivity in these animals. Our studies also suggest that ozone may produce similar effects.

The spread or metastasis of cancer is a very complex process which involves not only the properties of the cancer cell, but also the vasculature and defense system of the host. An adverse pollutant effect on any one of the cells in these compartments could facilitate progression of cancer. Therefore, the investigations should not be limited to the cells of the lung but should include other cell types and organs. Of particular value would be the studies of blood capillary lining cells of the lungs or kidney and the cells of the defense system. Epidemiological studies of disease processes where the underlying alterations are in the defense system or blood capillaries would be of great value. Studies on groups of people who have lived in a clean environment versus a polluted one could provide findings which could be correlated to basic experimental data from animal studies. There is a definite paucity of information about air pollutant effects on the immune system particularly in the light of modern immunology. Our preliminary studies with spleen cells have indicated that the immune system is an important area to be investigated. The immune system plays a role not only in cancer and infectious diseases, it is closely interrelated with many different biological processes in health and disease. The cells of the immune system and the blood capillary endothelial cells should be on

the priority list for investigations. The knowledge of pollutant effects on these major body systems should play a role in setting air quality standards.

8. BODY OF REPORT

Introduction

a. Scope and Purpose of the Project, General Background of the Project.

There have been a number of studies where NO_2 effects on different biological systems have been investigated and several studies have indicated adverse effects at the cellular and molecular level (1-6). Our studies have been focused on the role that NO_2 may play in the facilitation or enhancement of cancer cell dissemination and metastases development. The spread of cancer and the development of secondary cancer masses or metastases is the major complication which prevents the cure of cancer. Considering that there are at least 896,000 new cases of cancer annually in the United States, it must be considered as a significant health problem. Therefore, the importance of determining the influence of common air pollutants on the progression of cancer cannot be overemphasized and need careful evaluation. Particularly, the development of metastases from circulating cancer cells since inhalation of NO_2 could alter the host and lead to facilitation of metastases development. It should be pointed out that the existence of circulating cancer cells in cancer patients is well recognized (7,8). It is also known that most of the cancer cells in circulation are destroyed by the host unless there is an overburden of cancer or impairment in blood dynamics, integrity of vasculature or alterations in defense system (9,10). Our studies have indicated that inhalation of 0.3-0.8 ppm of NO_2 for a period of 10 weeks or longer will facilitate blood borne cancer cell metastasis to the lungs of exposed animals. These are the first reported studies which have linked NO_2 inhalation and facilitation of blood borne cancer cell metastasis - a new aspect of NO_2 inhalation hazards (11-13).

In view of the above described findings, four major objectives were set forth in this project: (1) to determine if combining the incidence of lung metastases with the incidence of metastases among animals would yield a more sensitive indicator for adverse NO_2 effects; (2) to determine if exposure to

0.4 ppm of NO_2 before cancer development (transplantation) would influence the growth and metastasis of these cancer cells; (3) to carry out preliminary studies towards identification of tissue and cellular changes which may be responsible for the facilitation of blood borne cancer cell metastases; and (4) to determine if exposure to 0.15 ppm of O_3 facilitates blood borne cancer cell metastases. The results from these studies should provide new information on the hazards associated with the inhalation of ambient or near ambient levels of NO_2 and O_3, and should be of assistance in making decisions on air quality standards.

b. Design, Materials and Methods.

An Overview

In three of the experiments of this project we used the same mouse melanoma metastasis model, as in our previous studies, except with fewer circulating cancer cells. In brief, five-week old C57 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 (14). In all experiments the NO_2 level was kept at 0.4 $\pm$ 0.05 ppm, a level which is often encountered in the Los Angeles area and was used in our earlier studies. The NO_2 and clean air exposures were carried out in special, identical environmental chambers. The desired NO_2 concentration was delivered to the NO_2 exposure chambers according to the method developed in this laboratory (15). The NO_2 concentrations were monitored by Saltzman (16) and chemiluminescence methodologies. Following an appropriate exposure, the animals of both groups were infused intravenously with viable melanoma cells. The melanoma cells were carried in tissue culture and single cell suspensions were prepared when needed for infusion. The surviving infused cells developed black melanoma 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 and control animals (clean air exposed) the effects

of NO_2 on the host can be assessed. In addition, there was a second model - a rat mammary adenocarcinoma. In this model the influence of exposure to 0.4 ppm of NO_2 on the growth and metastasis of transplanted cancer cells was studied. Finally, preliminary experiments were carried out to see if spleen cells of exposed and control animals can interact with cancer cells and influence the development of lung metastases.

Specific Experiments

1. Incidence of metastases in lungs and among animals exposed to 0.4 ± 0.05 ppm NO_2.

This experiment was designed to determine if the incidence of lung metastases and the incidence of metastases among animals could be used to indicate the adverse effects of NO_2. In the past only the incidence of lung metastases in the control and exposed animals has been compared. In this experiment there were 34 animals (C57BL/6J male mice) in control and NO_2 exposed groups. The animals were exposed for five days per week, seven hours ± one hour per day for a total of 435 hours (Table 6). Following this time period the animals were infused intravenously with 5×10^3 viable melanoma cells. Twenty-one days later the animals were killed by an intraperitoneal injection of 1 ml of pentobarbital (60 mg/ml). The lungs were removed en block and fixed via bronchial tree perfusion with 10% acetate buffered formalin. The lungs were then separated into lobes and the number of melanoma nodules per lobe was counted utilizing stereo microscope with 1X objective and 12.5 ocular. The data were analyzed using Mann-Whitney-U test. The findings are presented in the results section and summarized in Table 1.

2. Growth and Metastasis of Rat Mammary Adenocarcinoma, 0.4 ± 0.05 ppm NO_2 Exposure.

In this experiment the influence of NO_2 exposure on the growth of transplanted carcinoma cells was studied. The cell transplant into the mammary tissue represented a development of a primary cancer. Till this time we had studied only the NO_2 effects on bloodborne cancer cell growth.

The experiment had 32 animals in the control group and 33 in the exposed. The animals were exposed 5 days a week for 7 hours ± 1 hour per day for a total of 554 hours (Table 7). Following 427 hrs of exposure, the control and exposed animals were anesthesized and implanted with 10^6 cancer cells. The cells were suspended in 0.5 ml of tissue culture media and injected into the mammary fat pad. Following cancer cell implantation the animals were returned to their respective exposure chambers and the exposure continued. Twenty days later 10 animals from each group were killed to determine the growth and progression of cancer at this time. The remaining animals were killed 26 days postcancer cell implantation. The implantation site, lungs, liver and para-aortic lymph nodes were examined for cancer cell metastasis. The results are presented in the results section and summarized in Tables 2 and 3. The x^2 statistics were used to analyze these data.

3. Animal Exposure to 0.15 ppm of O_3 and Blood Borne Cancer Cell Metastasis to the Lungs.

The aim of the experiment was to evaluate ozone effects using the same biological probe as for NO_2. There were 55 mice in the control and O_3 exposure groups. Exposure was carried out 5 days a week for 7 hours ± 1 hour per day for a total of 419 hours (Table 8). Three exposed animals died during this period. Following the exposure period all animals were weighed and infused via the tail vein with 2.5×10^3 viable melanoma (B16F10R3) cells in 0.2 ml of balanced salt solution. Following cell infusion the animals were kept for three weeks in a filtered air environment. After this time period the animals were weighed again and killed with intraperitoneal injection of 1.0 ml of pentobarbital (60 mg/ml). The animals were then dissected and the lungs removed en block, inflated with buffered formalin and stored in the same. At a later date the lungs were separated into lobes and each lobe was examined for melanoma nodules using stereo microscope as before. The spleens were also removed from the animals and weighed. The results are presented in result section and summarized in Table 4. Mann-Whitney statistical analyses was used.

4. Animal Exposure to 0.4 ppm of NO_2 and the Spleen Cell Responsiveness.

Preliminary Experiments were carried out to determine if impairment in the immune system (spleen cells) could be detected. It was aimed to detect NO_2 effects on the natural killer (NK) cells of the spleen since it has been shown that these cells play a significant role in controlling bloodborne cancer cell spread. Cell suspensions were prepared from 4 spleens of exposed and control animals from experiment #1. The exposed and control spleen cells were then mixed in separate tubes with melanoma cells in a ratio of 400 spleen cells (mononuclear) to one melanoma cell. No attempt was made to separate different cell types. The mixtures were incubated at 37°C for 4 hours with gentle mixing every half hour. Following the incubation period the respective cell mixtures were injected intravenously into 17-week old mice which were pretreated 3 days earlier with 240 mg cyclophosphamide/kg of body weight. There were two groups of 10 mice each. One group received melanoma cells treated with exposed animal spleen cells; and the other group received melanoma cells treated with control spleen cells. In addition, 10 of the NO_2 exposed mice received melanoma cells treated with exposed spleen cells. Thirty-four control and exposed animals received untreated melanoma cells. Three weeks later the animals were killed as before and the lungs were evaluated for melanoma nodules. The results are presented in the results section and summarized in Table 5.

c. Results

1. Incidence of metastases in the lungs and among animals exposed to 0.4 ± 0.05 ppm NO_2.

A summary of the data is presented in Table 1. It can be seen that on the average the exposed animals had 4 nodules per lung and controls had 2.5 nodules. The difference is statistically significant ($P < 0.05$). There is also a significant difference in the number of animals among control and exposed groups who developed metastases. In controls we had 79% of the animals with metastases and in the exposed group we had 94%. In other words, there were only 6% of the animals in the exposed group without metatases while controls had 21% without metastases.

2. Growth and Metastases of Rat Mammary Adenocarcinoma, 0.4 ± 0.05 ppm NO_2 exposure.

Twenty days postimplantation of cancer cells, 10 animals from the control and exposed group were sacrificed. It was expected that at this time 100% of the control animals would show growth of carcinoma at the implantation site and that 35% will show lung metastases. The findings were different, however, and are summarized in Table 2. Eighty percent of the exposed and 60% of the control animals had carcinoma at the implantation site. No metastases were detected in the controls, but 3 of the positive exposed animals had lung metastases and 2 of these also had lymph node metastases. There were no significant differences between exposed and control groups according to x^2 statistics. Because of these findings the remaining animals were exposed an additional 6 days to see if more controls would develop cancer growth at the site of implantation. The results from this latter period are summarized in Table 3. At this time 78% of the exposed animals and 77% of the controls had shown growth of carcinoma. Fourty-one percent of the controls showed lung metastases and 54% had lymph node metastases. In the exposed group there were 28% of the animals with lung and 39% with lymph node metastases. The differences were not statistically significant.

3. Exposure to 0.15 ppm O_3 and Metastases Development.

In the control group 49% of the animals had melanoma nodules in their lungs compared to 58% of the animals with positive lungs in the O_3-exposed group. Exposed animals had 2.4 melanoma nodules per positive lung while controls had 2.0 nodules per positive lung (Table 4). Another finding was the significantly lower average body weight in O_3 exposed animals, following exposure, 26.63 gm. vs. 27.68/gm. ($P < 0.005$, student's t-test).

4. Spleen Cell Effects on Cancer Cell Growth.

In this preliminary experiment three groups of 10 animals were used. The animals were pretreated with cyclophosphamide to eliminate or reduce the

animals own immune response and were then injected intravenously with cancer cells which were treated with exposed animal spleen cells or control animal spleen cells. The group of animals which received cancer cells exposed in vitro to control spleen lymphocytes developed on average 74 melanoma nodules per animal. The group receiving melanoma cells pretreated with NO_2 exposed animal spleen lymphocytes developed 28 melanoma nodules per lung. The difference is highly significant, $P < 0.005$. The exposed animals receiving melanoma cells pretreated with exposed spleen cells developed 10 nodules/lung. The exposed and control animals, injected with untreated melanoma cells had 4 and 2.5 nodules per lung respectively (Table 5).

d. Discussion

The experiments carried out under this contract were designed to investigate NO_2 (0.4 ppm) and O_3 (0.15 ppm) effects on the host and how such effects in turn could influence the spread of blood born cancer cells. While in our previous studies only the effects of NO_2 were studied, utilizing blood borne cancer cells, the experiments under this contract also included studies of O_3 and NO_2 effects on growth and spread of transplanted mammary adenocarcinoma. The transplanted cancer cells were used to simulate a primary cancer growth. In addition, preliminary studies were carried out to see if alterations in the immune system, particularly in spleen lymphocyte populations, could be detected and correlated with facilitation of cancer cell metastases development. The overall objective of the project was to obtain new information about adverse NO_2 and O_3 inhalation effects utilizing an animal model which has relevance to human disease.

In one of the experiments the goal was to improve the sensitivity of the method by using low number of blood borne cancer cells (2.5×10^5) as a probe to reveal adverse NO_2 effects. It is pertinent to point out that it is known that under usual conditions only 0.1% of blood borne cancer cells will survive and progress to metastases development (17). Therefore, we were looking for a considerable adverse effect on the host in order to observe a higher incidence of metastases with such a small number of cells. The data has indicated that on the average exposed animals had 4.0 melanoma nodules per lung and controls had 2.5 nodules, a statistically significant

difference. There was also significantly higher percentage of animals in the exposed group with lung metastases. In other words, only 6% of the animals in the exposed group without metastases while in controls 21% of animals were free of metastases. The number of metastases per lung was low, compared to our other experiments, but this most likely reflects the low number of injected cells. It is possible that the differences could have been greater with slightly higher inoculum. However, the experiment permitted us to demonstrate increased incidence of lung metastases in exposed animals as well as increased number of animals in the exposed group with metastases. With respect to the latter, this experiment adds another dimension to our studies since now we have demonstrated not only the increased incidence of lung metastases but also increased incidence among animals in the exposed group which provides more sensitive means for measuring pollutant effects.

The results from the experiment, where we tested the NO_2 (0.4 ppm) effect on the growth and metastases of transplanted mammary adenocarcinoma, are difficult to interpret since the controls did not behave as predicted from the data in literature. It was expected that 100% of controls will develop adenocarcinoma at the site of inoculation and 35% of these would metastasize to the lungs. The increased incidence of lung metastases again would indicate adverse NO_2 effects. It was determined that at 20 days posttransplantation 60% of controls and 80% of exposed animals had growing mammary adenocarcinomas and 37% of exposed animals had metastases to lungs or lymph nodes. There were no metastases observed in the control group. At a later date there were no differences in transplant takes between controls and the exposed animals but the control group animals had more metastases than the exposed group.

The data were really too limited to make definitive interpretation. However, some possibilities should be pointed out. First, the lack of 100% growth in control group may be due to the age of animals since all previous transplantation studies had used 5 week old animals while in this experiment due to the exposure time (12 weeks) the rats were transplanted at age of 17 weeks which may make the difference. It is of interest that at 20 days posttransplantation only the exposed animals had metastases in lungs. It

appears that an early growth of transplanted cancer cells was induced by NO_2 exposure of these animals. This could be similar to increased early mortality from metastases which we have observed in NO_2 exposed mice in our earlier studies (18). Thus we may be detecting a trend indicating adverse NO_2 effect. Expanded studies in this area could provide more definitive results and answers.

In the ozone experiment the animals were exposed to 0.15 ± 0.02 ppm, the level which is just slightly higher than the state of Calfifornia standard. The incidence of metastases development was higher among O_3 exposed animals by 9%, but this difference is not statistically significant. However, it is of interest that the exposed animals at the end of experimental period had significantly lower average body weight ($P < 0.005$). The latter observation implies there was a significant O_3 effect on these animals. The mechanisms responsible for this are not known and only additional studies could shed light on this subject. The lack of metastases facilitation could be related to the low number of cells infused (2×10^3), it would be advisable to carry out additional experiments with more cells. Keeping in mind that only 0.1% cells are expected to survive we probably did not provide enough cells to detect the adverse effects.

Preliminary bioassay for natural killer cells was also carried out. The recipient animals were unexposed, healthy mice. They were pretreated with cyclophosphamide to eliminate or to suppress their own immune reactivity. Following this procedure, group of 10 animals were injected intravenously with melanoma cells which were pretreated in vitro with exposed animal spleen cells or control spleen cells. The main control was the group injected with untreated melanoma cells. The lymphocyte treatments should affect the viability of melanoma cells if the lymphocytes aren't affected by the NO_2 exposure. On the other hand if the lymphocytes were altered, there should be more metastases. The results were somewhat unexpected since both treatment groups developed significantly more metastases than the animals injected with untreated melanoma cells. It was of particular interest that melanoma cells treated with unexposed animal spleen cells, produced the highest incidence of metastases. The reason for this is not clear at the present time. Additional work will be needed to

exclude technical error before interpreting the results. However, this experiment has demonstrated the feasability of this approach to test spleen cell functions and it has shown that NO_2 exposed animal spleen cells affect the melanoma cells differently than the control spleen cells. This also implies that there is a NO_2 effect on spleen cells but further studies are needed to sort out the details and mechanisms involved in order to make a proper interpretation.

In general, one can say that the results from the experiments performed under this contract provide additional evidence that the inhalation of NO_2 or O_3 at ambient levels induce harmful effects. In addition, the experiments suggest that a great complexity of mechanisms may exist which are responsible for the observed results. Our model, where we utilized blood borne cancer cells as a probe to detect adverse effects could have great biological significance and implications on similar events in the human population.

TABLE 1

Incidence of Melanoma Nodules in Lungs and among animals, 0.4 ± 0.05 ppm NO_2 exposure, 435 hrs.

Treatment	No. Animals	% Animals with Metastases	MNo. Nodules/lung	Mann-Whitney Probability
Filtered Air	34	79	2.5 (0-11)	$p < 0.05$
NO_2	34	94	4.0 (0-12)	

At the beginning of the experiment the animals were 5 weeks old.

TABLE 2

Growth and Metastasis of Rat Mammary Adenocarcinoma, 20 days postimplantation, 0.4 ± 0.05 ppm NO_2 exposure, 508 hrs.

Treatment	No. Animals	carcinoma growth	metastasis lung|ly.n		x^2-Fisher test
Filtered Air	10	6	0	0	NS
NO_2	10	8	3	2	

NS-not significant
ly.n-lymph nodes

At the beginning of the experiment the animals were 5 weeks old.

TABLE 3

Growth and Metastasis of Rat Mammary Adenocarcinoma, 26 days postimplantation, 0.4 ± 0.05 ppm NO_2 exposure, 554 hrs.

Treatment	No. animals	carcinoma growth	metastasis lung|ly.n		x^2-Yates test
Filtered Air	22	17 (77%)	7(41%)	12(54%)	NS
NO_2	23	18 (78%)	6(28%)	9(39%)	

NS-not signficant
ly.n-lymph nodes

At the beginning of the experiment the animals were 5 weeks old.

TABLE 4

Incidence of Lung metastases in animals exposed to 0.15 ± 0.02 ppm O_3, 419 hrs.

Treatment	No. Animals	Metastasis	MNo. Metastases/lung	Mann-Whitney probability
Filtered Air	55	27	2.4	NS
O_3	52	58	2.0	

At the beginning of the experiment the animals were 5 weeks old.

TABLE 5

Spleen cell effect on metastatic potential of blood borne melanoma cells.

Recipient Treatment	No. Animals	Melanoma cell treatment	M Melanoma nodules/lung	Mann-Whitney probability
Filtered Air	34	None	2.5	$p < 0.05$*
0.4 ppm NO_2	34	None	4.0	
0.4 ppm NO_2	9	x-splc	10.0	
Filtered Air + CPH	9	x-splc	27.0	
Filtered Air	8	c-splc	74.0	

CPH - cyclophasphamide

* $p < 0.05$ or more significant in all cases.

x-splc - NO_2 exposed animal spleen cells, 12 weeks NO_2 exposure

c-splc - control animal spleen cells

All animals were 17 weeks old.

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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:

1. A. Richters, V. Richters and W.P. Alley.
   The mortality rate from lung metastases in animals inhaling Nitrogen dioxide (NO_2).
   J. Surg. Oncology, in press, 1984.

2. K. V. Kuraitis and A. Richters.
   Biphasic spleen changes following exposure to ambient levels of NO_2.
   Toxicology Letters, - submitted, 1984.

11. APPENDICES

Copies of the manuscripts are included as appendices A and B.

Appendix A

BIPHASIC SPLEEN CHANGES FOLLOWING EXPOSURE TO AMBIENT LEVELS OF NO_2

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Key words: NO_2, Spleen weights, Splenocyte hyperplasia

Acknowledgements: The authors wish to acknowledge the assistance of Drs. Valda Richters and Russell P. Sherwin

Supported in part by contracts A6-218-30, A2-128-33 from the State of California Air Resources Board and EPRI = RP1437-1.

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Summary

The effects of ambient levels of NO_2 (0.25 - 0.30 ± 0.05 ppm) on the spleens were evaluated. The results have indicated that after 6 weeks of NO_2 exposure the spleens were significantly enlarged in NO_2 exposed animals and following 12 weeks exposure the spleens were significantly smaller in the NO_2 exposed group. During the 4 and 10 week post exposure periods the spleens returned to near nromal values. It is concluded that the spleen enlargment after 6 week exposure was due to spleen cell hyperplasia while the small spleens of NO_2 exposed animals after 12 weeks may represent hypoplasia. Thus the spleen showed biphasic response to ambient level NO_2 inhalation exposure. It remains to be determined if the hypoplasia is reversible.

Introduction

Numerous studies have shown that the inhalation of NO_2 can produce both functional and structural changes in the lungs of experimental animals [1,2]. Although it is important to evaluate the effects of air borne pollutants on pulmonary tissues, studies designed to observe the consequences of such pollutants at extrapulmonary sites are equally important. This is emphasized by recent reports indicating that inhaled NO_2 and its subsequent reaction products are spread hematogenously from the lungs to other organs [3-5]. In view of this data, the important question of whether inhaled NO_2 affects organs other than the lung must be addressed in order to insure a thorough and appropriate appraisal of an air pollutant. Except for the effects of various circulating blood elements [6-8] and some immunologic studies [9-12] very few studies have investigated the effects of NO_2 on tissues other than the lung [13-16].

The spleen is of particular interest since it is so intricately related to circulating blood elements and plays an important immunological role. Limited reports have indirectly implicated altered splenic responses following NO_2 exposures by demonstrating depressed *in vitro* responses of spleen T and B cells to mitogenic stimuli [11]. In addition, graft rejection and immunoglobulin production was altered following NO_2 exposures [9].

With respect to direct in vivo observations of the possible splenic involvement following NO_2 exposures, one report simply mentioned that there were no microscopically observable changes in splenic histology following NO_2 exposures [13]. However, we have recently demonstrated a significant increase in spleen weights following ambient levels of NO_2 exposure for 6 weeks. In addition, the use of computerized image analysis enabled us to demonstrate subtle changes in splenic lymphoid nodule architecture [17].

This current study was conducted in an attempt to determine whether such spleen changes persisted at longer exposure periods and whether the changes were reversible after the experimental animals were allowed recovery periods in clean filtered air.

Methods

Animal Exposure to NO_2

In each experiment the animals were divided into two groups and housed in identical environmental chambers having a common filtered-air intake. A predetermined concentration of NO_2 gas was supplied to the air inflow of the experimental chamber, as was described previously [18]. The gas level was continuously monitored with a Teco chemiluminesence NO_2 analyzer and a Beckman analyzer, using Saltzman fluid. In addition, at least two weekly NO_2 gas level checks ere performed with a fritted bubbler by the technique of Saltzman [19].

The experiments described were conducted at NO_2 levels of 0.25 ± 0.05 or 0.30 ± 0.05 ppm. The control animals received filtered clean air. The NO_2 gas was delivered to the experimental chamber for 8 hours per day, 5 days per week, for a period of 8 or 12 weeks and the experiments were designated M120 and M116 respectively. In another experiment (M117) the 6 weeks of NO_2 exposure was followed by two recovery periods, one of 4 weeks and the other of 10 weeks, during which the animals received clean filtered air. following each exposure and recovery period equal number of animals were terminated from each group and lungs and spleens were removed. A total of 169 newborn male Swiss/Webster mice were studied in these experiments.

Only the effects on the spleens are covered in this report, the results from the lung observations will be reported elsewhere [20]. In these experiments, gravid mice were placed in the chambers 2 weeks prior to delivery and the pups were born in the chambers so that they would receive the maximal exposures at the earliest age possible.

Spleen Weight Assay

Following the various time periods in the environmental chambers, equal numbers of control and exposed animals were removed from the chambers, weighed and killed in a sequential order of matched pairs of control and exposed animals with a 0.5 ml i.p. injection of sodium pentobarbital. The lungs were removed en bloc and preserved for further studies. The spleens were dissected free of adherent tissue, placed in Petri dishes and were weighed immediately. The weights were expressed as a percent of the body weight (percent spleen weight). The data were statistically analyzed using Student's t test.

Results and Discussion

Table 1 presents data which demonstrate that changes in the percent spleen weight (%SW) are dependent on the dose of exposure to NO_2. Our previous report demonstrated a significant increase in the %SW following 6 weeks of low level NO_2 exposure [17]. Following a longer exposure period (12 weeks) at the same low level of NO_2, the exposed group of animals had a significantly lower ($p<0.01$) %SW. The relatively equal %SW observed in both the exposed and control groups at the 8 week test period could reflect a dynamic transition from larger to smaller spleens--a biphasic response. This data additionally mandates the exercise of caution when interpreting the biologic effects of NO_2 inhalation following single isolated exposure periods. It is not unreasonable to assume that increased exposure lengths at a given NO_2 level could have different effects on the same organ system. With respect to the spleen, the initial increase in spleen weight and size of lymphoid nodules [17] at 6 weeks of NO_2 may indeed be a physiologic splenic response to subtle acute pathologic changes in the lung. With longer exposure periods, the spleen itself may become a target organ with subsequent changes in function. Preliminary data from our laboratory [21,22] suggest that the altered spleen weights may reflect changes in spleen cell numbers and lymphocyte subpopulations. More extensive observations of these preliminary findings are being currently pursued in our laboratory. Furthermore, such spleen changes may account for the altered spleen cell functions as reported by others [9,11]. Both suppression and enhancement of various immune functions have been reported. It is interesting that the respective experiments were carried out at different NO_2 exposure lengths. Our results lend support to this biphasic pattern of responsiveness.

Moreover, NO_2 effects on spleens were also observed in a recent study employing nucleic acid hybridization techniques to investigate the expression of endogenous viral RNA in the lungs, thymus, kidney, and spleen of animals following various exposure lengths to NO_2 at near ambient levels [23]. It is important to point out that following 5 and 6 week test periods, only the spleen demonstrated a significant increase in endogenous viral expression.

In addition, Table 1 also provides data demonstrating that at 4 and 10 weeks of clean air exposure following 6 weeks of NO_2, the increased spleen weights in the exposed groups returned to weights equal those of the control groups. This reversibility may be interpreted as the resolvement of a physiologic response which was due to an acute NO_2 insult on the host. Unfortunately, clean air recovery periods following longer exposure periods when the spleens are decreased in size have not yet been conducted.

Spleen changes in response to the inhalation of a gaseous air pollutant should warrant considerable attention when assessing the overall health effects of ambient level NO_2 inhalation. The role of splenic immune functions in human health can be appreciated from morbidity and mortality reports with respect to asplenic individuals and fulminant pneumococcal disease [24-26]. In addition, the severest symptoms and some fatalities during the recent babesiosis outbreak in Nantucket occurred in splenectomized patients [27,28]. Therefore, altered splenic function could

provide an appropriate milieu for the expression of infectious diseases as well as microbial opportunism. Support that this indeed may be the case is suggested by studies reporting decreased resistance to infections in animals following NO_2 exposures [29,30].

In conclusion, it can be stated that the inhalation of ambient levels of NO_2 can influence the %SW and most likely reflects functional changes of the spleen as well. These splenic changes appear to express themselves in a biphasic manner and the enlarged spleens may normalize following extended clean air periods. However, more information is needed with respect to the smaller spleens at extended NO_2 exposure periods since it suggests detrimental effects on the spleen itself. The latter is supported by our current preliminary studies on spleen cellularity (Kuraitis, unpublished observations). The specific cellular changes in the spleen which may be responsible for the %SW changes are under investigation at the present time.

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Table 1

Percent spleen weights of newborn Swiss/Webster male mice exposed to NO_2 at 0.25-0.30 $\pm$ 0.05 ppm and the respective controls at various test periods, ie, 6,8, and 12 wks. Two recovery periods of 4 and 10 weeks following 6 wks of NO_2 are also presented.

Experiment number	Treatment	Exposure length in wks	CONTROL Mean % spleen wt ± S.D.	EXPOSED Mean % spleen wt ± S.D.	t-test significance
M117	0.30 ± 0.05ppm NO_2	6*	0.479 ± 0.094 (15)	0.596 ± 0.099 (15)	$p<0.0025$
M120	0.30 ± 0.05ppm NO_2	8	0.400 ± 0.046 (16)	0.429 ± 0.067 (15)	$p<0.1$
M116	0.25 ± 0.05ppm NO_2	12	0.473 ± 0.094 (37)	0.418 ± 0.099 (41)	$p<0.01$
M117	.30 ± 0.05ppm NO_2 recovery in clean air	6 + 4	0.447 ± 0.102 (15)	0.450 ± 0.068 (15)	NS
M117	.30 ± 0.05ppm NO_2 recovery in clean air	6 + 10	0.347 ± 0.056 (15)	0.359 ± 0.044 (15)	NS

( ) DENOTES NUMBER OF ANIMALS USED

SD STANDARD DEVIATION

NS NOT SIGNIFICANT

* DATA FOR 6 WEEK TEST PERIOD PRESENTED PREVIOUSLY (17) AND SHOWN HERE TO APPRECIATE AND COMPARE SUBSEQUENT ADDITIONAL DATA.

Appendix B

The Mortality Rate From Lung Metastases in Animals Inhaling Nitrogen Dioxide (NO_2)*

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Acknowledgements: The authors wish to acknowledge the assistance of Dr. Russell P. Sherwin and the El Monte Division of the California Air Resources Board.

Key words: mortality, cancer metastasis, air pollutants

SUMMARY

A study was carried out to determine the interrelationship between the inhalation of nitrogen dioxide (0.4 ± 0.05ppm), lung metastases development from circulating cancer cells and death rate from such metastases. C57 BL/6J mice were used in these experiments. Animals were divided into control and NO_2 exposed groups, and were exposed to filtered air and 0.4 ppm of NO_2, respectively. Following 12 weeks of exposure, all animals were infused intravenously with syngeneic, viable B16 melanoma cells. The results indicate that a subpopulation of NO_2 exposed animals showed a significant increase in mortality rate during the early part of the experiment. The interpretation is that animals especially sensitive to the NO_2 insult developed extensive metastases at an early stage. The question raised is whether or not the progression of human cancer is influenced by the inhalation of noxious pollutants in the ambient atmosphere.

INTRODUCTION

Secondary cancerous growths in lung tissues or pulmonary metastases usually result from blood borne cancer cells [1]. It is also known that several extrinsic and intrinsic factors may produce conditions in the host which favor the development and progression of metastases [2-4]. The best documented of these are immune suppression, capillary endothelial cell alterations, cancer cell homotypic or heterotypic aggregations, cancer cell interactions with components of the blood clotting system, and tissue damage in general [5-9]. Some of these conditions may arise from inhalation of common air pollutants [10-13] but little attention has been given to the possibility that inhalation of air pollutants may enhance the spread and dissemination of cancer. However, we have recently demonstrated that inhalation of ambient and near anbient levels of NO_2 or ambient air pollution facilitates the development of lung metastases from circulating cancer cells (mouse melanoma). Mice exposed to NO_2 and those breathing ambient air developed significantly greater numbers of melanoma nodules in their lungs than animals inhaling clean air [14,15].

In this report we present data from a preliminary study indicating that a number of NO_2 exposed animals with lung metastases exhibit increased mortality rates. This is the first time that inhalation of 0.4 ± 0.05 ppm of NO_2 has been linked to increased blood borne cancer cell metastasis to the lungs and accelerated death rate.

MATERIALS AND METHODS

Five week old C57 BKL6J male mice were used in this experiment. Animals were divided into two groups, i.e. NO_2 exposed and control. There were 48 animals in each group and they were kept in identical environmental chambers. The control animals received filtered air free of NO_2 and O_3 and those exposed to NO_2 received the same filtered air supplemented with 0.4 ± 0.05 ppm NO_2.

The NO_2 was delivered to the chamber according to a previously described method [16,17] and the levels were monitored continously with the chemiluminscence NOX analyzer and a Beckman instrument utilizing Saltzman fluid. In addition, a fritted bubbler was used weekly to check NO_2 levels according to Saltzman's methodology [18]. The total No_2 exposure was 429 hours. This was spread over a 12 week period with a daily average exposure of 7 ± 0.5 hours for five days a week. Water and food were supplied to the mice ad libitum.

Following the 12 week exposure, animals of the two groups were infused via tail vein with 10^5 single cell suspension of viable melanoma cells. One mouse in the NO_2 group was runt and was not used. The cell line B16F10R2 was used, which was established in our laboratory from a pulmonary melanoma nodule which developed in a C57Bl/6J male mouse following intravenous infusion of B16F10R1 cells. The B16F10R2 cell line is carried in tissue culture according to the previously described protocol [19].

Following cell infusion, NO_2 exposure was discontinued and the control and NO_2 exposed groups were supplied filtered air. Twenty-one days later 10 mice from each group were killed by an overdose of pentobarbital (60mg/ml) injection to determine if metastases were developing. The lungs were removed en bloc, inflated with acetate buffered formalin and examined by stereomicroscopy for melanoma nodules as previously reported [19]. All remaining animals were observed daily and the dead animals were necropsied to determine the extent of metastasis.

RESULTS

The examination of mouse lungs at the 21 day post-melanoma cell infusion revealed that control mice had an average of 31 melanoma nodules in their lungs and NO_2 exposed mice an average of 83 nodules. The nodule count of the control and NO_2 exposed groups ranged from 3 to 83 and from 1 to 214, respectively, with a standard deviation of 29.28 for controls and 85.76 for the NO_2 exposed group. The difference was not statistically significant, but it must be stressed that on

average the NO_2 exposed mice had 50 or more melanoma nodules per lung than control mice.

Since the first death occurred on the 29th day after melanoma cell infusion, the survival curves were constructed beginning with day 28. At that time, there were 38 control nd 37 NO_2 exposed mice. Mortality in the NO_2 exposed group was highest during the first 7 days of the survival period. This was then followed by a period during which the mortality rate was lower among NO_2 exposed animals and during the very last part of the survival period no deaths occurred among NO_2 exposed animals.

Therefore, the mortality rates were examined according to the time intervals reflecting the aforementioned trends (Table 1). The data were divided into 3 representative time periods and were evaluated by regression analysis and analysis of covariance [20].

For the first time period (days 29-36) the mortality rate for No_2 exposed mice was a -2.58 mice per day while for controls it was -1.17, and represented a significant difference at the 1 percentile level (F=21.15). The analysis of covariance indicated that the average number of survivors in control group (33.75) was significantly greater than in NO_2 exposed group (30.38) at the 5 percentile level.

There was no significant difference between the mortality rates in the second time period (days 37 to 46, Table 1). The mortality rate during this period for the NO_2 exposed mice dropped and approached the control value, while the mortality rate of control mice increased as expected. The average number of mice surviving in the control group for this period (11.50) was still significantly greater at the 1 percentile level than that of the NO_2 exposed mice (7.80). None of the mice exposed to NO_2 died in the third time period (days 47-53). The animals of the control group continued to die at a reduced rate.

Necropsies revealed that death was due to a progression of lung metastases followed by massive involvement of the lungs, thoracic cavity, and mediastinum.

At the end of the 53rd day, there were one control and two NO_2 exposed mice alive. There were no deaths observed during the next seven days and the experiment was terminated. The necropsies of these animals showed that the control mouse had no metastases. One of the NO_2 exposed mice had a melanoma nodule on the tail at the injection site and no metastases in the lungs. The other NO_2 exposed mouse had several large nodules in the lungs and liver.

DISCUSSION

The spread of cancer or metastasis is one of the major complicating factors which often prevents successful elimination of a particular cancer. As we have already indicated, several extrinsic factors may influence the course of metastasis by affecting the host. Our studies, therefore, have been focused on NO_2, a common environmental air pollutant. In comparing the mortality rates of the control and NO_2 exposed animals, a significant difference was found during the first part of survival period. This increased mortality rate in our opinion reflects increased metastases formation and progression in the mice most sensitive to NO_2 insult. Since the mortality rate in NO_2 exposed group during the second time period was not significantly different from the control group, and dropped to zero in the 3rd period, it is very likely that all animals with increased sensitivity were lost during the first period. The difference in sensitivity among inbred mice should not be a surprising since different radiation sensitivity has been observed in this and other mouse strains in establishing LD_{50} [21] and variations in biological responsiveness are recognized [22].

Although this is a limited study, the finding of an increased mortality among NO_2 exposed animals may have relevance to human cancer since it is well recognized that some humans are especially sensitive to air pollutants [23]. The recognition of especially sensitive animals, where the incidence of lung

metastases and mortality rate may be increased by a preventable insult (in this case the inhalation of NO_2), should be of concern. It further emphasizes the hidden risks that may be associated with the inhalation of ambient or near ambient levels of NO_2. Thus, the inhalation of noxious air pollutants at ambient or near ambient levels may have a much greater impact on cancer progression than recognized at the present time. The epidemiological studies correlating increased cancer mortality with polluted urban environment [24,25], and the increased incidence of melanoma metastasis in smokers [26], may be indicative of inhaled pollutant effects on the spread of human cancer.

The mechanisms responsible for metastasis facilitation are not clear at the present time and future studies are planned to address this question.

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Table 1

Statistical Summary of Survivors and Mortality Rates

Groups	REGRESSION ANALYSIS: Average Survivors	REGRESSION ANALYSIS: Regression Coefficient (Mortality Rate)	ANALYSIS OF COVARIANCE: Homogeneity of Regression	ANALYSIS OF COVARIANCE: Analysis of Covariance
		1st Period (Days 29-36)		
Control	33.75	-1.17	F = 21.15**	F = 8.97*
NO_2 Exposed	30.38	-2.58	DF = 1,12	DF = 1,13
		2nd Period (Days 37-46)		
Control	11.50	-1.86	F = 0.87 NS	F = 21.51**
NO_2 Exposed	7.80	-1.60	DF = 1,11	DF = 1,17
		3rd Period (Days 47-53)		
Control	3.28	-0.61	F = 46.61**	F = 10.15**
NO_2 Exposed	2.00	0.00	DF = 1,10	DF = 1,11

* (Significant at 5%)

** (Significant at 1%)

NS (Not Significant)