FINAL REPORT

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THE ROLE OF NO_2 AND O_3 IN CANCER METASTASIS AND IN SYSTEMIC ADVERSE EFFECTS

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1. ABSTRACT

In recent years, we have utilized a unique biological probe (living cancer cells) to detect NO_2 and O_3 induced adverse effects. The indicator for adverse effects has been the increased incidence of melanoma nodule development in the lungs from the intravenously injected cancer cells. Our past studies have demonstrated that inhalation of 0.3 - 0.8 ppm of NO_2 will facilitate melanoma cell metastasis to the lungs of exposed animals, but exposure to ozone (0.15 ppm) does not exhibit this phenomenon. In experiments carried out under this contract we have demonstrated that exposure to a combination of NO_2 and O_3 at 0.35 ppm and 0.15 ppm, respectively, results in facilitation of blood-borne melanoma cell metastasis. It appears that in this system NO_2 may be the more harmful component. Of the many possible cellular systems that NO_2 may affect, the defense system and the lung blood capillaries would be the most likely targets, and damage to these cells could account for these findings. Our immunological studies of the defense system have indicated the following: 1) spleen T lymphocytes, T-helper and T-supressor cells showed a trend towards lower percentages of these cells after 12 weeks of NO_2 exposure and 2) the functional test for Natural Killer (NK) cells indicated suppressed cytotoxicity following 6 weeks of exposure. The lung capillary lining cells of exposed animals showed a greater degree of cell injury as revealed by ultrastructural studies and increased number of thrombi in lung capillaries. Alterations in these two systems would also imply systemic NO_2 effect with a number of possible adverse consequences. Even though these studies were carried out in an animal model, 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 a subclinical disease process. The

knowledge of pollutant effects on different cells of the defense system could provide a sensitive indicator for adverse effects and should play a role in 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 CONCLUSION

It is well recognized that inhalation of air pollutants can have adverse affects on many biological systems and functions (1,2,3). The severity and the long term impact on health would depend upon duration of exposure and the specific air pollutant involved. Of particular importance is the knowledge of ambient level exposures since such levels are encountered by a large number of people in urban areas. It is also realized that an adverse effect can have several different levels of biological significance (4), and if the effect is associated with a major disease process or alteration in a major biological system, it will be of major concern to health investigators and environmental control agencies. In view of the foregoing, we have utilized a unique biological probe (living cancer cells) to detect NO_2 and O_3 induced adverse effects. The indicator for adverse effects has been the increased incidence of melanoma nodule development in the lungs from intravenously injected cancer cells. Our past studies have demonstrated that inhalation of 0.3 - 0.8 ppm of NO_2 will facilitate melanoma cell metastasis to the lungs of exposed animals, but exposure to ozone (0.15 ppm) did not exhibit this phenomenon. The studies under this contract have indicated that exposure to combination of NO_2 and O_3 at 0.35 ppm and 0.15 ppm, respectively, results in facilitation of blood borne melanoma cell metastasis. It appears that in this system NO_2 may be the more harmful component. Of the many possible cellular systems that NO_2 may affect, the defense system and the lung blood capillaries would be the most likely targets, and damage to these cells could account for these findings. Our immunological studies of the defense system have indicated the following: 1) spleen T lymphocytes, T-helper and T-suppressor cells showed a trend towards lower percentages of these cells after 12 weeks of NO_2 exposure, and 2) the functional test for NK cells indicated

suppressed cytotoxicity following 6 weeks of exposure. The lung capillary lining cells of exposed animals showed a greater degree of cell injury as revealed by ultrastructural studies. The small capillary thrombi (clots) associated with endothelial cell injury showed entrapment of circulating cancer cells. Thus, the most important aspect of these experiments is the fact that NO_2 inhalation, by affecting different host parameters, can enhance or facilitate the dissemination of cancer, particularly circulating cancer cells. Since most cancer patients have cancer cells in their blood at some time during the course of cancer progression (5), these findings have direct bearing on people with cancer, keeping in mind that one out of three persons will develop cancer during his lifetime (6). Moreover, alterations in the immune sytem and blood capillaries would also imply systemic NO_2 effects with a number of possible adverse consequences. Efforts should continue to identify substances in air which may lead to significant impairment of biological systems and health.

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 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 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 and other adverse effects. Therefore, investigations should not be limited to the cells of the lung but should include other cell types and organs. 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, since it is closely interrrelated with many different biological processes in health and disease. The cells of the immune system, particularly the lymphocyte subtypes 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.

The presence of pollutants in the environment, especially those with carcinogenic properties, has been of great concern to environmental health scientists and air quality regulatory agencies. In view of this, many studies have been directed toward the identification of cancer causing agents in the air we breathe (7-10). None of these studies, except our own recent investigations, discussed below, has considered the possibility that exposure to certain air pollutants could enhance cancer cell spread or metastasis. The problem of cancer involves not only the development and presence of cancer 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 (11,12). Considering the fact that a significant segment of the population in the United States is already affected by cancer and that the probability is one in three that an individuals will develop cancer (6), a question arises as to the role of environmental air pollutants in the dissemination of cancer cells and the development of metastases. 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 (13)."

Since the presence of circulating cancer cells is well recognized in cancer patients (13-18), it is important to identify extrinsic factors which may affect the host and influence the survival of cancer cells and development of metastases. In general, the conditions which may arise and favor the development of cancer cell metastases from circulating cancer cells 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 (19-23). It is known that some of these conditions may also result from inhalation of noxious air pollutants (24,25). With respect to the latter, there are epidemiological studies which suggest that there is increased mortality from cancer in polluted urban areas (26,27) which would imply increased incidence of metastases, but there are also reports which contradict this (28,29). Most importantly, none of the mentioned studies has addressed the question of incidence or frequency of metastases development among people living in polluted areas in spite of the fact that it is the most important and destructive phase in the progression of cancer. There is only one reported study which specifically shows increased incidence of metastases in patients with malignant melanoma who have had a history of smoking (30), which we consider a personal pollution effect.

Since the major destructive action of cancer is due to disseminated cancer cells or cancer metastasis, it is important to prevent or delay this process as much as possible. Therefore, it is necessary to identify the substances in the environment which may influence the spread and progression of cancer by affecting specific host cells and organs.

In view of the foregoing, for the last six years we have studied the effects of NO_2 inhalation on blood borne cancer cell

spread to lungs in an experimental mouse melanoma metastasis model. Using this model, we have been looking only at one stage of the complex process of metastasis, ie, the stage when cancer cells are present in the blood stream, the critical step in dissemination of cancer cells. Our studies in the last six years have produced several publications dealing with this subject and we have shown the following:

1. Intermittent inhalation of NO_2 at the levels of 0.3 - 0.8 ppm for a period of 10 weeks or longer facilitates blood borne cancer cell metastasis to the lungs of exposed animals. The exposed animals develop significantly greater number of melanoma nodules in their lungs (31,32).

2. The melanoma nodules progress and a significant number of exposed animals die sooner than the controls (33).

3. Inhalation of Los Angeles ambient air (Vivaria room air) facilitates blood borne cancer cell spread to lungs just as does exposure to 0.4 ppm NO_2 alone (34).

It must be emphasized at this point that even though we have demonstrated the adverse NO_2 and ambient air effects as indicated by increased incidence of lung metastasis in exposed animals, the specific reasons for the observed effect, are still unknown.

B. Design, materials and methods.

An Overview

Two major objectives were set forth for the project described in this report:

1. To determine the effects of ambient level NO_2 and O_3 mixtures on the incidence of metastasis development, since the existence of such mixtures in an urban environment is well recognized.

2. To identify specific cells and tissues which have been affected by NO_2 to the extent that facilitation of cancer cell metastasis takes place.

The first objective was accomplished by exposing animals to NO_2 (0.3 ppm) and O_3 (0.15 ppm) mixture for 12 weeks. Following this period, the animals were infused intravenously with melanoma cells as in our previous experiments, and 3 weeks later the lungs and other organs (liver, spleen, kidney, intestines) were examined for melanoma nodules. The lungs were separated into respective lobes and the number of melanoma nodules observable on the surface of each lobe was recorded utilizing stereomicroscope. The surface nodules of other mentioned organs were recorded for the entire surface area. Increased incidence of melanoma nodules in exposed animals would indicate an adverse effect caused by the inhalation of the gas mixture.

To fulfill the second objective, the cells of the immune system and blood capillary lining cells (endothelium) were studied. With respect to the immune system, the number of T lymphocytes in spleens of exposed and control animals were

compared and the spleen NK cell function was assessed by an in vivo bioassay utilizing melanoma cell survival and growth as an indicator. It has been demonstrated by other investigators that, in general, the suppression of spleen NK cell activity will result in increased incidence of experimental metastases in lungs from circulating cancer cells (35,36). The NO_2 effects on NK cells have never been tested. This is a new approach which could provide valuable new indicators for adverse air pollutant effects.

Specific Experiments

Experiment No. 1. Exposure to NO_2 (0.35 $\pm$ 0.05 ppm) and 03 (0.15 $\pm$ 0.02 ppm) mixture.

This experiment was designed to determine if animal exposure to NO_2 and 0_3 mixture facilitates the blood borne cancer cell metastasis to lungs and other organs. Five week old C57Bl/6J male mice were used in this study with 50 animals in control and exposed groups. Following a 420 hour exposure period, animals of both groups were infused intravenously with 10^4 viable melanoma cells. Three weeks later all animals were sacrificed and the lungs and other organs were examined for melanoma metastases as indicated above. The data were evaluated using an appropriate statistical analysis. A bioassay for NK cells was also carried out as described below.

Experiment No. 2. Exposure to NO_2 (0.4 $\pm$ 0.05 ppm).

This experiment was designed to test the following:

1) NO_2 effect on immune system, particularly the NK cell function, utilizing in vivo spleen NK cell bioassay. The enumeration of spleen T lymphocytes also was carried

out utilizing specific fluorescine labeled monoclonal antibodies.

2) NO_2 effect on lung capillary lining cells (endothelium), utilizing ultrastructural examination.

3) Platelet aggregation and clot formation in the capillaries of the lungs utilizing light and electron microscopy.

Five week old C57Bl/6J male mice were used in this experiment with 150 mice in control and experimental groups. The NO_2 exposure was for 210 and 420 hours and following each exposure period the above mentioned testing was carried out as described below.

An experiment was also designed to study macrophage function utilizing the blood carbon clearance test as indicator. However, serious difficulties were encountered in obtaining uniformed blood samples at proper time intervals. This resulted in very irregular results which were impossible to interpret. In view of this the test was discontinued following the trial with the first twenty animals. Instead, the study of lung capillaries by light and electron microscopy was expanded which appeared to be a more promising approach.

1) Animal exposure to air pollutants. The control and exposed animals were housed in identical environmental chambers having a common filtered (Purafil) air intake. The desired concentration of NO_2 was introduced into the experimental exposure chamber via the air intake by the method which has been described in detail previously (37) and is in use at the present time in this

laboratory. The level of NO_2 was continuously monitored with a Teco chemiluminescence NO_2 analyzer and a Beckman analyzer using Saltzman fluid. In addition, at least two weekly NO_2 gas level checks were performed with a fritted bubbler employing the technique of Saltzman (38). The NO_2 gas was delivered to the exposure chamber for 7 hours/day $\pm$ 1 hour, 5 days/week for a total of the specified exposure period for each experiment.

The ozone delivery system consisted of an ozonisator (Sander Model IV) into which 100% oxygen was delivered at a flow rate of 8-20 ml/min depending on the level desired and with the output of the ozonisator kept constant at 30%. A control system for oxygen flow rate was placed in series with the ozone monitor (Dasibi) to prevent a rise in the ozone level above the planned upper limit. Calibration of the Dasibi and other monitoring instruments was carried out by the California Air Resources Board technical staff (El Monte).

2) Melanoma cell infusion. B16F10R4 melanoma cell cultures were carried in this laboratory according to previously established protocol (31). In brief, all tissue cultures were maintained in plastic tissue culture T flasks in RPMI-1640 medium supplemented with 10% fetal calf serum. The medium was changed twice weekly and the cultures were transferred once a week. For experimental needs the cells were collected from the flasks using Ca^{++} and Mg^{++} free Earles's balanced salt solution (EBSS) containing 0.025% EDTA. The single cell suspensions were then washed with culture medium and resuspended in EBSS. The animals were restrained and cells of the desired concentration in 0.2 ml were

infused via the tail vein.

3) Evaluation of melanoma nodules in the lungs. Following the prescribed exposure periods, the animals were killed by an overdose of pentabarbital, the lungs were removed and inflated with 10% acetate buffered formalin and stored in the same. The melanoma nodules (black) were visible on the surface of each lobe of the lung and were counted utilizing the stereomicroscope. It should be pointed out that the metastatic potential of cells changes with continued propogation in vitro. Due to this the number of melanoma nodules developing from a given number of injected cells will vary among experiments performed at different times. This precludes comparisons of the actual numbers of melanoma nodules among assays performed at different times. Only the relative percent differences between matched experiments can be compared keeping in mind the age of the animals and the biological variability. Histopathological sections of the lungs were also prepared for pathological evaluation. The collected data from the exposed and control animals were analyzed by an appropriate non-parametric statistical analysis.

4) T lymphocyte enumeration in the spleens. Ten control and ten exposed spleens were used following each NO_2 exposure period. The spleen cell suspensions were prepared from freshly removed spleens using 60 mesh stainless steel screen and rubber policemen. Each spleen suspension was treated separately. The cell suspensions then were centrifuged and stained with fluorescein conjugated antibodies. The specific antibodies used were anti-mouse Thy-1.2, anti-Lyt-1 and

anti-Lyt-2. The stained cells were counted in a fluorescence activated cell sorter (FACS).

5) Bioassay for spleen Natural Killer (NK) cells. The spleens from ten NO_2 exposed and ten control animals were tested following 210 and 420 hour exposures. The cell suspensions were prepared using stainless steel 60 mesh screens as described above. The cells from the exposed and control animals were pooled separately and counted. The lymphoid cells were then put on a monolayer of melanoma cells in ratio of 50 lymphocytes to one melanoma cell and incubated for four hours at 37°C. Following incubation the lymphocytes were washed off, the melanoma cells removed and resuspended in EBSS and 0.2 ml of the cell suspension was injected intravenously into five week old C57Bl/6J male mice. There were 20 five week old recipient mice for each treatment group. The recipient animal pretreatment with cyclophosphamide was omitted in this study, since preliminary studies under previous contract has indicated that use of this compound may lead to unpredictable and unexplainable animal responses. This bioassay for melanoma cell ability to proliferate *in vivo* should be superior to ^{51}Cr release cytotoxicity assays *in vitro* since it has been shown that NK cells may render melanoma cells cytostatic without lysis (39).

6) Ultrastructural studies of lung endothelium. Following each exposure period and after infusion of 1×10^5 cancer cells in ten animals from each group, five animals from each group were sacrificed at 4 hours and one day after cell infusion. The lungs from these animals were removed en block, perfused via bronchial

tree with 2.5% glutaraldehyde in cacodylate buffer. After initial fixation the tissues were processed by the routine electron microscopy method which is used in this laboratory (40). In brief, the method includes postfixation in osmium, dehydration in graded alcohols, clearing in propylene oxide and embedding in epon. This was followed by cutting thick sections (luM) from lung periphery and thin sections (500A) from selected areas of thick section. The thick sections permit evaluation of relatively large area of lung. The general structure of lung can be evaluated but thin sections are needed for individual cell and subcellular evaluation. Changes in cell junctions and cell surface integrity can be observed only with electron microscopic evaluation of thin sections. The main value of thick sections is to identify areas for thin section using and to detect some changes in capillaries such as thrombi. The cellular nature or makeup of the thrombus is studied utilizing the thin sections and electron microscope. The thin sections were stained with uranyl acetate and lead nitrate and mounted on grids for observation in Phillips electron microscope.

C. RESULTS

1) Incidence of metastases in the lung of animals exposed to a mixture of 0.35 + 0.05 ppm NO_2 and 0.15 ppm O_3 for 12 weeks. A summary of data is presented in Table 1. It can be seen that the animals exposed to NO_2 and O_3 mixture developed significantly higher number of melanoma metastases in the lungs. Two animals had inadequate cell infusions and were not included in statistical analysis.

2) Bioassay for Natural Killer (NK) cell activity following 12 weeks NO_2 and O_3 exposure. A summary of data is presented in Table 2. It can be seen that the melanoma cells treated with exposed animal spleen cells, the source of the NK cells, produced significantly more colonies than in untreated animals. This indicates that NK cell cytotoxic or cytostatic ability was suppressed by NO_2 and O_3 exposure.

3) Bioassay for NK cell activity following 6 and 12 weeks of 0.4 + 0.05 ppm NO_2 exposure. A summary of data is presented in Table 3. It can be seen that 6 weeks of NO_2 exposure apparently affected NK cells adversly as indicated by higher number of melanoma nodules developed

in the bioassay animals. However, the 12 week exposure yielded more nodules from cells treated with control animal spleen cells. The reason for this reversal is not clear at the present time.

4) Quantitation of spleen T lymphocyte subtypes. A summary of the data is presented in Table 4. The results indicate a trend towards decreased percentages of T cells in exposed animals. However, the animal numbers are small, and no definite conclusion can be made.

5) Capillary lining cell evaluation.

a) **Light microscopy.** The one micron thick sections were used to evaluate the clot formation in capillaries. A total of 125 such sections were scored at 4 and 24 hours after melanoma cell injection, following 6 and 12 weeks of NO_2 exposure. The incidence of lung capillary thrombi after six weeks of NO_2 exposure is higher in exposed animals. The same is also true following 12 week exposure but the difference is smaller. Even though the differences are not statistically significant, these findings indicate that there is injury of capillary endothelial cells beyond regular turnover of cells in exposed animals. A summary of data

is presented in Table 5.

b) **Electron microscope.** A total of 126 thin sections (500 A^{o}) were studied by electron microsocpe and 107 representative pictures were taken. The pictures have revealed lung capillary endothelial cell injury. The injury consists of "blisters" on endothelial cells and in some areas, complete lack of endothelial cells, exposing the basement membrane. The latter is probably responsible for platelet adhesion and clotting in those areas. Electron microscopic examination of thrombi revealed several instances where melanoma cells were entrapped in platelets. Since these sections are very thin it is not surprising that only in relatively few instances the melanoma cells or their processes were noted. Only complete serial sectioning of all thrombi would assure presence or absence of cancer cells as discussed below. This type of injury was observed in both control and exposed animals. The injured areas in exposed animals appeared to be more extensive; there were bigger blisters and larger areas of endothelial denudation. It appears that the incidence of injury did not increase beyond 6 weeks of exposure.

D. DISCUSSION

The major objective of our studies has been to determine which cells and tissues in the body may be affected adversly by inhalation of ambient concentrations of NO_2 and O_3. We chose to use blood borne cancer cells as a biological probe to detect adverse effects. The increased incidence of metastases in the lungs of exposed animals would indicate adverse pollutant effect. Since cancer metastasis and wide dissemination is often associated with immune suppression, capillary endothelial cell alterations, changes in blood clotting mechanisms and tissue damage in general (19-23), increased metastasis in exposed animals could mean that pollutants induced one or more of the above conditions. Because of this possibility, the studies described in this report were intended to examine the most likely possibilities, that is, the effects on cells of the immune system and the lung capillary lining cells.

Our earlier studies have demonstrated that inhalation of NO_2 in a concentration range of 0.3 - 0.8 ppm facilitated metastasis, while concentrations of 0.3 and 0.15 ppm of O_3 did not show this phenomenon (32). However, in our experiment described in this report, when we used a mixture of 0.35 ppm NO_2 and 0.15 ppm of O_3, significant facilitation of blood-borne cancer cell metastasis developed. It is difficult to say whether there was a synergism of the two gases, because there is considerable variation from experiment to experiment in the number of melano nodules developing. The exposed animals may show 50-130 % increase in melanoma nodules. There are several reasons for this variability: 1) the metastatic potential of melanoma cells may change during in vitro cell propogation; 2) the age and natural susceptibility of animals play a role and 3) unknown factors influence cell functions during in vitro manipulations. In view of this the actual numbers of cancer nodules can be compared only among

experiments carried out at the same time, with the same pool of cells, keeping in mind the animal age and biological variability. However, the relative percent increase in different experiments may provide some insight. In the experiment with NO_2 and O_3 mixture there was 70% increase in nodules in exposed animals and this increase is within a range observed with NO_2 only. With respect to the defense system, limited studies were carried out to evaluate spleen lymphocytes and NK cells. The bioassay for NK cells revealed that 12 week exposure to this mixture apparently suppressed the cytotoxic capabilities of NK cells or reduced their numbers. This affect is reflected in increased numbers of melanoma nodules developing from melanoma cells treated with exposed animal spleen cells (Table 2). Similar results were also observed when melanoma cells were treated with spleen cells from NO_2 exposed animals following 6 weeks of exposure (Table 3). The spleen cells from 12 week exposure did not show this effect. One has to realize that the number of animals in these experiments was small, and caution must by exercised in interpreting these data. However, the biological implications of these findings are so great that they warrant preliminary publication and more expanded studies.

The T lymphocyte subtypes were also quantitated to detect air pollutant effects. The results have shown overall lower T lymphocyte population in spleens of exposed animals (Table 4). The specific T lymphocyte subtypes, identified by Lyt-1 and Lyt-2 antigens, showed lower values than controls, but the difference was not significant. Again, one has to use caution in interpretations since we were dealing with low numbers of animals and single experimental results. However, these are important areas for future studies of air pollutant effects. This was the first attempt to see if air pollutants affect the T lymphocyte subtypes. The T lymphocyte subtypes play an important role in different immune responses.

Studies at the ultrastructural level have revealed injury to capillary endothelial cells in the lung. Even though these findings were not quantitative they provide correlation and a structural basis for earlier observations in this laboratory which demonstrated leakage of blood proteins from lung capillaries (41).

Moreover, it is known that injury to capillary lining cells enhances circulating cancer cell extravesation and metastasis. The major mechanism is probably through clot formation and entrapment of cancer cells. The demonstration of increased incidence of capillary thrombi with entrapped cancer cells in NO_2 exposed animals provide supporting evidence that these events may, in part, be responsible for facilitation of metastasis.

The most important aspect of these experiments is the fact that NO_2 inhalation, by affecting different host parameters, can enhance or facilitate the dissemination of cancer, particularly the circulating cancer cells. These findings are highly relevent to human health problems because alterations in the immune system or blood capillaries, the focus of these studies, are frequently associated with diverse human diseases. If indeed these or similar events are taking place in humans, as some epidemiological studies tend to suggest (26,27), the control of NO_2 levels in urban areas may be critical to human health. The exposure may lead not only to increased and accelerated dissemination of cancer but to several other serious health problems, such as enhancement of atherosclerosis development, decreased resistance to viral and bacterial infections and expression of other opportunistic organisms. In general, one can say that the results from experiments under this contract provide new evidence that the inhalation of 0.3 ± 0.05 ppm NO_2 and 0.15 ± 0.003 ppm of O_3 induce significant harmful effects in animals. Whether humans are affected similarly remains to be established. Nevertheless, one has to wonder if present air quality standards are adequate.

Table 1
Incidence of Melanoma Nodules in Lungs of NO_2 and O_3 Exposed Animals

Treatment	No. Animals	$\bar{M}$ No. Lung	S.D.	Mann-Whitney Probability
Filtered Air	48	64.00	54.50	0.0354
NO_2 (0.35 ppm) O_3 (0.15 ppm) 12 weeks	50	89.66	60.10	

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

S.D. - standard deviation

Table 2
Incidence of Melanoma Nodules in Lungs of Unexposed Animals[+] - NK Cell Bioassay

Animal Treatment[*]	No. Animals	$\bar{M}$ No. Lung	S.D.	Kruskal-Wallis Probability
Filtered	8	48.63	32.20	
$NO_2 + O_3$ (0.35 ppm + 0.15ppm) 12 weeks	8	102.50	51.34	0.05

[*]Spleen cells of these animals were the source of the NK cells.
[+]15 weeks old C57BL/6J mice.

$\bar{M}$ - mean number of nodules per lung
S.D. - standard deviation

Table 3
Incidence of Melanoma Nodules in NK-Bioassay Animals

Animal Treatment Providing Splenic Cells*	No. Animals	$\bar{M}$ Nodules Lung	Mann-Whitney Probability
Filtered Air, 6 weeks	15	3.0	p = 0.0096
0.35 ppm NO_2, 6 weeks	19	12.8	
Filtered Air, 12 weeks	20	78.7	p = 0.0000
0.35 ppm NO_2, 12 weeks	19	22.2	

*Splenic cells used as a source of NK cells.

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

Table 4
Splenic T-lymphocyte Population
Percent Fluorescence

Animal Treatment	No. Animals	Thy 1.2*	S.D.	T-lymphocyte Markers Lyt-1	S.D.	Lyt-2	S.D.
Filtered Air	9	45.3	10.0	38.6	12.5	17.4	6.7
Nitrogen Dioxide 0.35 ppm ± 0.05 ppm for 12 weeks	10	36.6	9.4	35.0	7.8	15.9	5.5**
t test		p = 0.0679		NS		NS	

Thy 1.2 - All T-lymphocytes
Lyt-1 - Helper/inducer T-subset
Lyt-2 - Suppressor T-cells
*T-test: 45.3 vs 37.4, p = 0.0679
NS - not significant
** only 9 samples

Table 5
Incidence of Capillary Thrombi

Treatment	No. animals	Ave. No. per mm_2 4 hs	S.D.	24 hs	S.D.	M.W. Test
Filtered Air	5	0.778	0.832	0.518	0.301	NS
NO_2, 0.35 ppm 6 weeks	5	0.956	0.652	1.018	0.511	
Filtered Air	5	0.654	0.553	0.634	0.362	NS
NO_2, 0.35 ppm 12 weeks	5	0.800	0.900	0.772	0.300	

hs - hours after cell injection

MW - Mann Whitney U test

NS - not significant

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11. APPENDIX

A copy of a submitted manuscript is included as Appendix A.

Appendix A

Blood-borne Cancer Cell Dissemination, Natural Killer (NK) Cells, and Air Pollution

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Abstract

Our studies have emphasized the identification of extrinsic factors, particularly environmental ones that may enhance metastasis. Using an animal model, we have demonstrated that inhalation of nitrogen dioxide (NO_2), a common air pollutant, can facilitate blood-borne cancer cell metastasis to lungs. In this report we present data which suggest that NK cell activity may be suppressed by inhalation of ambient levels of NO_2 and may, in part, explain the NO_2 facilitation effect. It also means that NO_2 induces biologically significant systemic adverse effects and cancer patients living in polluted environment may be at a great disadvantage. Quantitation of T suppressor and T helper lymphocytes was also carried out.

Key Words

Cancer, metastasis, defense, air pollution.

Introduction

The ability of cancer cells to metastasize and to grow progressively leads to destruction of vital organs and death of the host. Such cancer progression depends upon cancer cell properties and many intrinsic host conditions and factors (1,2). In addition, several extrinsic factors, such as radiation or drugs, can influence the host and the spread of cancer. Moreover, there is also a large number of environmental pollutants to which cancer patients may be exposed every day, and very little is known how these agents may affect the cancer patient and the progression of cancer. With respect to the latter, there are some epidemiological studies that suggest that the incidence of death from cancer is higher in polluted urban areas (3,4) and that smoking (personal pollution) may enhance the spread of malignant melanoma (5). The findings could be interpreted to indicate that pollutants in urban air enhance progression and dissemination of cancer cells by affecting the host. In view of the foregoing, we have tried to study the effect of nitrogen dioxide (NO_2), a common air pollutant, on blood borne cancer cell dissemination in an experimental animal system. Our findings have indicated that inhalation of ambient or near ambient concentrations of NO_2 facilitate blood-borne cancer cell spread to lungs of exposed animals (6,7). These animals develop significantly larger numbers of lung metastases and die sooner than the control animals (8). The mechanisms responsible for the facilitation is not clear at the present time. In this report we present data from a preliminary study which suggests that NO_2 may effect NK cells and their suppressed activity may, in part, be responsible for the observed NO_2 facilitation effect. Limited T-cell sub-population quantitation data is also presented.

Materials and Methods

Animal Exposure to NO_2

In all experiments, five week old C57BL/6J male mice were used. Animals were divided into two groups: NO_2 exposed and controls. The NO_2 was delivered to the chamber according to previously described method (9) and the levels were monitored continuously with chemiluminescence and 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 (10). The NO_2 concentration for this experiment was 0.35 ± 0.05 PPM, and the exposure was carried on for 12 weeks, 7 hours a day, for 5 days a week. The control animals received filtered air. Water and food were supplied to the mice ad libidum. Following the 6 week exposure period, animals from the control group and the exposed group were infused with B16F10R4 melanoma cells for NK cell assay as described below. The T lymphocyte subpopulations were evaluated following 12 weeks of exposure.

Bioassay for NK Cells

Spleen cell suspensions were used as a source of the NK cells. Five freshly dissected spleens were made into cell suspensions from exposed and control animals, utilizing number 40 mesh stainless steel screen and a rubber policeman. The red cells were lysed using distilled water method. The resultant cell suspension was passed through the stainless steel screen, the lymphoid cells then were washed in Eral's Balanced Salt (EBS) solution and suspended in growth medium, supplemented with 10% heat inactivated fetal calf serum. To test for NK cells, the lymphoid cell suspensions of exposed

and control animal spleens were added to separate T flask cultures containing monolayers of B16F10R4 mouse melanoma cells. This melanoma cell line had been developed from the original B16F10 cell line by passing the cells 4 more times through animal transplantation and in vitro cultures. The spleen cell and melanoma cell ratio was approximately 20 to 1 respectively. The cultures were incubated for 4 hours at 37°C. Following this time period the cultures were washed free of lymphoid cells, the monolayer cells were removed utilizing EDTA, washed and suspended in calcium and magnesium free EBS solution. Cells were tested for viability utilizing trypan blue dye. All melanoma cells showed 95% - 97% viability. The cells were then resuspended to have 10^4 cells per 0.2 ml of balanced salt solution for injection. Unexposed animals were injected with 10^4 viable cells in 0.2 ml of EBS solution via tail vein. There were three groups of animals: the first group received untreated cells, the second group received cells treated with exposed animal spleen cells and the third group received melanoma cells treated with control animal spleen cells. Three weeks later the animals were sacrificed. The lungs were removed enblock and inflated with buffered formalin. The dissecting microscope was used to examine the lungs and to count the melanoma nodules.

T Lymphocyte Evaluation

Fluorescein conjugated monoclonal antibodies to Thy 1.2, Lyt 1 and Lyt 2 were used to identify T cells, T helpers and T suppressors respectively. The spleen lymphoid cell suspensions from 10 control and 10 NO_2 exposed animals were studied. The lymphoid cells from each spleen suspension were suspended in RPMI-1640 media with 2% heat inactivated horse serum and

divided into 3 tubes. Each tube was stained with 5 microliters of appropriate antibody for 15 minutes on ice. Following staining, the cells were washed with EBS solution and resuspended in EBS for counting with Fluorescence Activated Cell Sorter (FACS).

Results

The examination of mouse lungs 21 days postmelanoma cell infusion revealed that the animals receiving untreated melanoma cells had on average 16.9 melanoma nodules per lung. The animals receiving melanoma cells treated with control animal spleen cells and cells treated with exposed animal spleen cells had 3.0 and 12.8 melanoma nodules per lung, respectively. These results indicate that exposed animal spleen cells (source of NK cells) were less reactive or suppressed, permitting survival of more melanoma cells. Summary of the data is presented in Table 1. The percentage differences in T lymphocyte subpopulations are presented in Table 2. It can be seen that there is a trend towards lower percentages in each subgroup of lymphocytes for the exposed animals. The most significant change is in the total T cell number, when comparing controls versus exposed animals we get a p value of 0.0679. Since Lyt 1 is not completely restricted to single subpopulation but it can be found at low concentrations also on other T cell subgroups and on NK cells, it is not surprising that the percentages of Lyt 1 and Lyt 2 do not add up to percentage of 51.2. It is also of interest that the ratio of Lyt 1 to Lyt 2 or helpers to suppressors is similar in both groups which is 2.2.

Discussion

Metastasis is considered by many as the most difficult problem in management of cancer. Many factors can influence the progression and dissemination of cancer. But only recently it has been pointed out, in an animal model, that certain air pollutants play a role in facilitation of blood-borne cancer cell metastasis (7). That the same events may take place in humans is suggested by some epidemiological studies (4,5,11); however, others have not found such a relationship (12,13). One has to recognize that epidemiological studies designed specifically to correlate the incidence of metastasis with air pollutant exposure still need to be carried out. However, there are experimental data suggesting that NO_2 inhalation affects the immune system (14,15,16). Since the status of the immune system is often associated with the extent of cancer spread, it is only natural to determine if pollutant effect on certain cells of the immune system could be responsible for enhancement of cancer metastasis. In our earlier studies we observed significant spleen weight changes and qualitative spleen cell population shifts in NO_2 exposed animals (17,18). The study described in this paper presents quantitative data on spleen lymphocyte subpopulations and a functional status of NK cells. The suppression of NK or cytotoxic cell activity is based on the reduced ability of melanoma cells to survive and produce melanoma nodules in lungs, following in vitro treatment with NO_2 exposed animal spleen cells (Table 1). This is the first report linking suppression of NK cell activity by an air pollutant, and possibly effects on T cell subpopulations, to enhanced cancer cell metastasis. It should be pointed out that there have been few other reports investigating T and B lymphocyte responses to NO_2 exposure (19,20).

However, NO_2 effects on NK cells and T cell subpopulations has not been reported before. It would appear that the evaluation of specific T cell subpopulations and NK cell activities would provide more sensitive indicator of air pollutant effects, not only with respect to spread of cancer but as indicators for systemic adverse effects. It is important to emphasize that air pollutant effects on T cell subpopulations or NK cells could permit development of infections, expression of opportunistic organisms and could lead to several other disorders. The role of NK cells in elimination of circulating cancer cells has been described by several other investigators (21). We realize that the data of this study are limited and need expanded sutdies, but the nature of the findings, particularly with respect to NK cells, has great biological significance and implications. Since our environment is becoming more and more complex, increasing number of toxic substances appear and new approaches are needed for evaluating their effects on health. The approach presented in this study is only one step in that direction. Since one in three individuals may develop cancer in the United States, it is important to identify environmental agents which have potential to enhance progression of this disease. It will be of further interest to determine how the effects vary with length of exposure and how permanent or reversible are the induced changes.

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Table I

INCIDENCE OF MELANOMA NODULES IN LUNGS
ROLE OF NK CELLS

ANIMAL TREATMENT PROVIDING NK CELLS	NO ANIMALS	AVERAGE NUMBER OF NODULES		MANN WHITNEY U-TEST
		PER LUNG	PER LOBE	
FILTERED AIR	15	3.0	1.2	P=0.03
0.35 ppm NO_2	19	12.8	3.7	
MELANOMA CELLS 10^3, UNTREATED	19	16.9	4.2	P=0.02

TABLE II

SPLENIC T-LYMPHOCYTE POPULTATIONS
PERCENT FLUORESCENCE

ANIMAL TREATMENT	NO ANIMALS	T-lymphocyte markers		
		Thy1.2*	Lyt-1	Lyt-2
Filtered Air	9	45.3	38.6	17.4
Nitrogen dioxide 0.35 $\pm$ 0.05 ppm for 6 weeks	10	37.4	35.3	15.9

Thy-1.2 - All T-lymphocytes
Lyt-1 - Helper/inducer T-subset
Lyt-2 - suppressor T-cells
* t-test, 45.3 vs. 37.4, p=0.0679