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NITROGEN DIOXIDE EFFECTS ON PROGRESSION OF MOUSE LYMPHOMA, A BLOOD CELL MALIGNANCY

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3. ABSTRACT

The main objective of the study was to determine if an adverse nitrogen dioxide effect on host could be detected by studying the host's immune system and the development and the progression of a spontaneously occurring lymphoma, in a mouse model, which very closely resembles human malignancy. The nitrogen dioxide level was 0.25 parts per million. Following five varying exposure periods, equal numbers of age matched control and exposed animals were studied utilizing histopathological and immunological methods. A ten month survival study was also carried out. The major findings were as follows: 1) significantly more exposed animals survived in the survival study; 2) the control animals showed more extensive lymphoma process following varying exposure periods; 3) the exposed animals showed significantly lower percentages of total spleen T lymphocytes and two subpopulations, following 37 and 181 days of exposure. Taken together these findings indicate that exposure to 0.25 ppm NO_2 delayed the development of fulminant lymphoma and had an adverse effect on T lymphocytes. Therefore, in this study, the NO_2 exposure did not enhance the advancement of malignancy, it delayed it. However this was due to the unique nature of this malignancy which originates among the major class of cells in defense system - the T lymphocytes. This delay should not be mistaken as being beneficial to the host. In fact, it is quite the opposite, the NO_2 exposure has affected important lymphocyte subtypes and has prolonged and aggravated the disease process. It should be emphasized that the major cells of the immune system were affected by NO_2 levels which are frequently encountered in many urban environments and one has to be concerned about similar effects occurring in human urban populations.

4. ACKNOWLEDGEMENTS

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5. 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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8. SUMMARY AND CONCLUSION

Previous studies in this laboratory, using a different animal model, have indicated that the spread of blood-borne cancer cells was facilitated by inhalation of ambient levels of NO_2. The exposed animals developed significantly greater numbers of lung metastases and died sooner than the controls. Since this model did not fully represent human cancer metastasis, there was a need for a model which would more closely resemble the human situation. For this reason a spontaneously occurring blood-cell malignancy known as T lymphoblastic lymphoma was selected. This is an AKR mouse lymphoma model which is considered analogous to human disease and exhibits remarkable immunological and clinicopathological similarities with T-lymphomas in children and young adults. In view of this, an experiment was designed to study NO_2 inhalation effects on the natural course of this malignancy. The major objective was to determine if inhalation of 0.25 ppm NO_2 affects host parameters which may in turn influence the advancement of this spontaneously occurring malignancy. The advancement of malignancy was judged from survival times, histopathological examinations, and quantitation of spleen, thymus and peripheral blood lymphocytes. Five week old female mice with 150 animals per control and experimental group were used in the study. Using the same exposure and monitoring methods as in our previous studies, the experimental group was exposed to 0.25 ± 0.05 ppm NO_2 for 5 days per week, 7 ± 1 hr. per day, for periods of 37, 71, 111, 141 and 181 days. The control animals received filtered clean air. Food and water was provided <u>ad libitum</u>. Following each exposure period, 18 control and exposed animals were sacrificed and their thymuses, spleens, lymph nodes, lungs and livers were removed and prepared for microscopic histopathological evaluation. The lymphocyte subtypes from spleens and thymuses, and natural killer cells from spleens were quantitated using specific monoclonal antibodies and fluorescence activated cell sorter (FACS) analysis. In addition, blood samples were taken for white cell counts and smears were prepared from blood and bone marrow. Fifty animals from each group were used for the survival study which lasted ten months. Autopsies were performed on all animals who died from the natural progression of the lymphoma. The results of this experiment revealed four major findings: 1) the percent of animals surviving, following a ten month

study, was significantly greater in exposed group; 2) the microscopic tissue evaluation showed more extensive lymphoma process in the control group; 3) there were significantly more small lymphocytes in peripheral blood of exposed animals following 37, 71 and 111 days of exposure, while for the same time periods there were significantly more large lymphocytes in the control groups; and 4) there were significantly lower percentages of total T lymphocytes as well as Lyt 2 and L3T4 T lymphocyte subpopulations in exposed animals following 37 days of NO_2 exposure. Following 181 days of NO_2 exposure only the L3T4 lymphocyte subtype showed lower percentages than controls. Taken together, these findings indicate that the disseminated disease was taking hold earlier in control than in exposed animals, and that L3T4 lymphocyte subtype may be particularly sensitive to these exposure conditions. Thus in this study the NO_2 exposure did not enhance the advancement of malignancy, it delayed it. However, this was due to the unique nature of this malignancy which originates among the major class of cells in defense system - the T lymphocytes. This delay should not be mistaken as being beneficial to the host. In fact, it is quite the opposite. The NO_2 exposure has prolonged the suffering and has aggravated the disease process by adversely affecting the quantity, and probably the function, of the cells in the immune system. It indicates that NO_2 interfered with growth of lymphoma or, in other words, NO_2 delayed the development of fulminant lymphoma. This delay is most likely due to the NO_2 effect on the mitotic activity of malignant lymphocytes. It may be that fewer cells entered mitotic cycle, or a longer time period was required to complete the mitotic cycle. The lower percentages of specific subtypes following different exposure periods suggest a direct NO_2 effect on subtypes of lymphocytes and differential sensitivity or vulnerability of these cells. These findings also suggest that NO_2 may interfere with normal lymphocyte proliferation, particularly in view of the 37 day results at which time most spleen and thymus lymphocytes should exhibit normal properties. The normal and malignant lymphocytes often depend upon the same growth factors and proliferation controlling mechanisms. To provide a complete explanation for all of the findings of this experiment, a study combining quantitative and functional evaluation of specific lymphocyte subtypes is needed. However, this study strongly suggests that low level NO_2 exposure may induce significant alterations in the cells of the immune

system, particularly specific subtypes of T lymphocytes which play an important role in control of various infections and in development and spread of cancer. Since these quantitative changes were induced by NO_2 levels which are frequently encountered in many urban environments, one should be concerned that similar effects may be taking place in urban populations. In addition this study has indicated that new and sensitive approaches for evaluating immune system should be explored to obtain the best possible indicators for defining harmless or tolerable air quality.

9. 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 effects of NO_2 on the complex defense system have been of particular interest to us. The defense system is composed of immunological and non-immunological compartments with many different cell types, their subtypes and interactions. The defense system plays the central role in protecting our bodies from a number of insults including development of malignancies and great variety of infections. During the last few years, knowledge about the immunological compartment of the defense system has grown a great deal. In light of these modern immunological advancements, there is a definite paucity of information about air pollutant effects on the immune system. In this report we have presented for the first time the finding that NO_2 affects specific subtypes of lymphocytes - the central cells of immunological defense. These are very significant findings since different cells in this defense compartment have different functions, leading to different risks and impairments. Thus the cells of the immune system, particularly the lymphocyte subtypes and their specific functions should be on the priority list for future investigations. By utilizing the voluminous new immunological knowledge and the modern tools available, significant information could be gained about air pollutant effects which would not be recognized without such approaches.

8. BODY OF REPORT

Introduction

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

It is well recognized that inhalation of air pollutants can have adverse affects on many biological systems and functions (1,2). 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 (3), 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, many studies have been directed towards identification of cancer-causing agents in the air we breathe (4,5,6) because cancer is an important disease and it affects a significant segment of the population in the United States (7). The air pollutant effect on progression and metastasis of cancer, for the most part, has been neglected even though there are some indications that the incidence of cancer deaths is higher in polluted areas (8,9,10) and in individuals with "personal pollution" habits (11). Our studies in the past few years have indicated that the survival and spread (metastasis) of blood-borne or circulating cancer cells (melanoma) in experimental animals is influenced by inhalation of ambient or near ambient levels (0.3 - 0.8 ppm) of NO_2. The exposed animals developed significantly more cancer nodules in their lungs than the animals breathing filtered air (12,13,14). The cancer burden or mass was significantly increased in these animals, and they died sooner than the respective controls (15). These findings clearly show adverse NO_2 inhalation effects on some biological systems of the host. In more recent experiments, we have carried out some preliminary studies on suspected systems and our results suggest that NO_2 may induce quantitative or functional changes in some cells of the immune system (16). However, the model used in our earlier studies was restricted to one phase of cancer

progression, i.e. the spread of blood-borne cancer cells. In order to gain better understanding of the NO_2 effects in the human situation, we had to extend the study one step further and ask the crucial question - would NO_2 inhalation influence the natural progression of a cancer, i.e. invasion, metastasis and dissemination of a spontaneously occurring and growing cancer by affecting host parameters? In order to accomplish this, one had to insist that the natural course of the cancer in a model system is comparable to the course of a human cancer, i.e. it has to grow, invade, metastasize and lead to generalized dissemination and death. Unfortunately, to study these events of complete progression of a solid non-hematological tumor in an animal model is almost impossible. This is due to the fact that most of the spontaneous and even experimentally induced animal tumors do not fulfill the criteria of human cancers, i.e. they seldom metastasize (17,18) or kill the host by the latter process (19,20), and if they do, it is only under very restricted conditions. It has been pointed out that, in many instances, animal models have not been realistic with respect to human cancer, particularly the mouse mammary tumors and lung adenomas of strain A mice (21,22). However, some of the blood cell malignancies have great similarities to human disease. One such available model is AKR/cum mouse lymphoma, which is analogous to human lymphoma in children and young adults (23). This murine leukemia virus (MuLVs) associated lymphoblastic lymphoma occurs spontaneously in 10-15% at 1-6 months of age, and in 71-85% at 6-12 months of age in AKR/cum females, and the mean age of death from lymphoma is 7.6 months. This model would not be limited to a single phase (blood borne cancer cell metastasis) but would permit to evaluate different steps in natural progression of lymphoma. It will be possible to establish the NO_2 effects on the survival as well as lymphoma-free period in exposed versus control animals.

The other important part of this study was a more detailed quantitative evaluation of specific cell types in immune system. Our earliest studies of spleen weights and more recently the cells of the spleen have pointed to NO_2 induced alterations (24,25). It is not clear if these alterations are quantitative or functional. Both aspects should be investigated, but since the work load would be overwhelming, we chose the quantitative approach for this project. The major objective was to

determine if there are specific quantitative changes in lymphocyte subpopulations and if these changes can be correlated with the extent of lymphoma and NO_2 exposure. We are the first research group to emphasize lymphocyte subpopulations, particularly the T-helper, T-suppressor, and the natural killer cells in evaluation of air pollution effects. We consider these three cell categories to be the major cellular compartments in the immune system which are involved in body's natural defense against cancer and infectious diseases which may be affected by inhalation of specific air pollutants. It is important to realize that any quantitative or functional imbalance, acquired or inherited, among these subpopulations of lymphocytes could lead not only to enhanced progression of cancer but to a number of diseases and cellular disorders. A good illustration for this is AIDS, a disease associated with T-helper lymphocyte depletion (26), severe infections, and lymphomas. The NK cell defect in Chediak-Higashi syndrome (27) is another example, where the patients have an inherited NK cell deficiency, and high incidence of infections and lymphoproliferative disorders. Therefore, if strong quantitative or functional changes were found in lymphocyte subpopulations due to pollutant inhalation, the tests for these subpopulations could serve as biological indicators for adverse air pollutant effects. Such tests would be simple to perform on peripheral blood on human populations, since modern immunology has the necessary reagents and methods for this.

B. DESIGN, MATERIALS AND METHODS

An Overview

The proposed study dealt with a spontaneously developing and progressing malignancy, i.e. lymphoblastic lymphoma. Only a few spontaneous animal malignancies are available and because of this, the time table for the experiment was dictated by the course of this disease. We began with 300 5 week old AKR/cum female mice. The reason for choosing 300 animals is that this is the maximum number we were able to obtain at one time. The animals were separated in weight-matched control and exposed groups with 150 animals per group. There were 5 animals per cage and the cages were housed in stainless steel environmental chambers. The control chamber received clean filtered air, and the experimental chamber received the same air supplemented with NO_2 to give 0.25 $\pm$ 0.05 ppm level in the chamber air. NO_2 was monitored continuously with a Teco chemilumenescence instrument. A number of animals (randomly picked) from each group were sacrificed at different intervals during the experiment to monitor the cells of the immune system and the onset and progression of lymphoma. On basis of our past experiments, where we observed spleen weight changes at 6 and 12 weeks post NO_2 exposure (24), and on basis of literature indicating the time of onset of lymphoma in these animals (28), we selected 6 sequential time periods for evaluating the status of the immune system and the progression of lymphoma. However, due to the encountered death rate, only 5 periods were used. Specific cells of the immune system were quantitated utilizing fluorescence labeled monoclonal antibodies and the extent of lymphoma was determined microscopically from hematoxylin-eosin stained tissue sections of different organs and bone marrow smears. White cell blood counts and blood smears were also used. At each predetermined time period, animals were sacrificed from each group. A group of fifty control and exposed animals was used for the survival study which lasted 10 months. It was limited to ten months because of the availability of age matched animals and because the average life span for lymphoma animals is 7.6 months (28). All animals who died during this period were autopsied to confirm the lymphoma. The surviving animals were killed at the end of a 10 month period to evaluate the cells of the immune system. The data obtained

were analyzed by appropriate statistical methods as described below. Thus, the major objective of this project was to determine whether inhalation of 0.25 $\pm$ 0.05 ppm of NO_2 affected host parameters which influenced the progression and spread of spontaneous lymphoma. It was intended to answer the following specific questions:

1. Does lymphoma occur earlier in exposed animals?
2. Is the survival time shortened in exposed animals?
3. What is the extent of lymphoma at different time intervals following exposure?
4. Are there significant quantitative differences in total T lymphocytes, T-helper cells, T-suppressor cells and natural killer cell following different exposure periods?

(1) Animal Exposure to Air Pollutant

The control and experimental 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 chamber via the air intake by the method described in detail previously (29) and 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 (30). The NO_2 gas was delivered to the exposure chamber for 7 hrs/day $\pm$ 1 hr, 5 days/week throughout the experimental period.

(2) Histopathologic Evaluation of Lymphoma

Following 37, 71, 111, 141 and 181 days of exposure, groups of 18 animals were sacrificed by intraperitoneal injection of 0.5 ml pentobarbital (60mg/ml). The tissues were dissected, weighed, fixed in phosphate buffered formalin and processed by routine histopathological methods. The extent of lymphoma was determined microscopically from histological sections of the following tissues: thymus, spleen, lymph nodes, bone marrow, liver, and lung. The following criteria were used: Category I - all tissues examined were consistent with normal morphology; Category II - early lymphoma as indicated by lymphoblastic (lymphoma cells) foci in thymus and/or spleen and lymph nodes; Category III - advanced lymphoma as indicated by partial or complete replacement with lymphoma cells of one or more of the following organs: thymus, spleen or lymph nodes. Slight to moderate infiltration of perivascular spaces in lung or liver may be present; and Category IV - disseminated lymphoma as indicated by lymphoma replacement of thymus and/or spleen and lymph nodes and by heavy infiltration lung, liver or bone marrow. Blood smears were prepared from peripheral blood and stained with Wright's stain. The white cell counts were carried out on 100 ul of blood utilizing a Coulter Cell Counter and an appropriate diluent and were used to obtain additional information with respect to lymphoma spread.

(3) Quantitation of Lymphocytes

Half of a spleen from each animal was used to quantitate lymphocytes. The spleen cell suspensions were prepared from freshly removed spleens using a 60 mesh stainless steel screen and a rubber policeman. The cell suspensions then were centrifuged and cells were resuspended in buffer with 0.5% heat inactivated bovine serum. The cell suspensions were then split into three equal aliquots and each aliquot was stained by a specific monoclonal fluorescent antibody. Anti Thy-1.2 was used to identify all T lymphocytes, anti L3T4 was used to identify T-helper cells, and anti Lyt-2 to identify T-suppressor cells. After appropriate cell washing, the cells were counted by an automated Fluorescence Activated Cell Sorter (FACS). The cells were also counted according to their size (large, small) since the large size would represent the normal lymphocyte populations. The same procedure was used for quantitation of thymus lymphocytes.

(4) Quantitation of NK Cells

One of the aliquots from each of the prepared spleen cell suspensions was used for staining NK cells. The same general procedure was used as for lymphocytes, except that an antibody to asialo GM1, which permits identification of NK cells, was used. The counting of cells was carried out with FACS.

7. Data Analysis

In this study we obtained descriptive histopathological information and quantitation data. The quantitative data came from survival study, body and organ weight evaluation and lymphocyte population studies. The Mantel-Cox test was used for testing the equality of survival curves, assisted by BMDP software, UCLA. Body and organ weights were analyzed by Student's t-test and lymphocyte populations by Mann-Whitney u-test.

C. RESULTS

1. Body Weights

Body weights were determined following each of five exposure periods. The only significant difference between the control and exposed animals was observed following the last 181 day exposure period. The control animals weighed an average of 31.2g and exposed animals weighed an average of 29.3g. The difference was significant ($p<0.02$) by student's t-test.

2. Percent Spleen and Thymus Weights

Following each exposure period the percent of body weight for spleen and thymus was determined. No significant differences were detected between the groups (Tables 1 and 2).

3. Survival Study

The survival study involved 50 control and 50 exposed 5-week-old animals. The time period was limited to ten months because of the availability of age matched animals and because the average survival time of lymphoma animals is 7.6 months. At the end of this period 28% of the control group and 50% of the exposed group survived. Survival curves as determined by the BMDPIL computer program are presented in Fig. 1. Statistical analysis of these data, using the Mantel-Cox test of the same program, gave a p value of 0.02, indicating significantly higher percent survival in the exposed group (Table 3).

4. Histopathological Evaluation of Lymphoma

Following each exposure period, 18 animals of each group were studied. The microscopic evaluation of thymus, spleen, lungs, liver, lymph nodes and bone marrow, according to criteria described above, provided information for placing all animals in four categories, following each of the five different exposure periods (Table 4). Category I represents animals exhibiting normal tissue histology; categories II through IV represent

different stages of lymphoma development.

Following the first exposure period (37 days), none of the controls showed completely normal histology; 14 showed early lymphoma and four had advanced disease. In the exposed group, for the same time period, 10 animals showed no indication of disease, and eight had early lymphoma. A similar trend can be seen after 71 days of exposure. Following the 111 day period, major differences can be seen in categories II and IV. Following the last two exposure periods (141 and 181 days), advanced lymphoma (category III) was more prevalent among exposed animals, while disseminated lymphoma (category IV) was more prevalent in the control group; the latter was also true after the 111 day exposure.

5. Peripheral Blood Leukocyte Counts

The total peripheral blood leukocyte counts showed a considerable increase in both groups as the experiment progressed. The highest values in controls were observed following the 141 day exposure and in the exposed group following the 181 day period. The differences were not statistically significant (Table 5).

6. Peripheral Blood Polymorphonuclear Leukocyte (PMN) and Lymphocyte (L) Differential Counts

White cell differential counts were performed on peripheral blood smears from 18 control and 18 exposed AKR/cum mice per exposure period. Occasionally the blood smears were not suitable for evaluation, decreasing the number available per group. The total lymphocyte and PMN counts showed no differences between control and exposed animals (Tables 6 and 7).

When lymphocytes were broken down into two groups by size, small and large, exposed animals had significantly more small lymphocytes in the first three exposure periods (64.9% vs. 38.5%, p ==> 0 after 37 days; 33.7% vs. 23.0%, p <0.01 after 71 days; 27.7% vs. 9.4%, p ==> 0 after 111 days (Table 5). However, control animals had significantly more large lymphocytes in the first three exposure periods (41.3% vs. 14.9%, p ==> 0

after 37 days; 42.9% vs. 32.0%, p <0.02 after 71 days; 59.4% vs. 34.4%, p ==> 0 after 111 days (Table 8 and 9).

After 141 days of exposure, exposed animals still had more small lymphocytes than control animals (15.1% vs. 10.2%), and control animals had more large lymphocytes than exposed animals (54.8% vs. 51.7%); however, the differences were not statistically significant.

After 181 days exposure, no differences in the number of small and large lymphocytes were observed between control and exposed animals.

7. Spleen Lymphocyte Subpopulation Quantitation

In general, percentages of all T lymphocyte subtypes were lower in the exposed group following each exposure period. Statistically significant differences were observed between control and exposed groups after 37 and 181 day exposures and are summarized in Table 10. Following the 37 day exposure, percentages of total T lymphocytes (identified by Thy1.2 antigen), large T lymphocytes (identified by Lyt2 antigen), and small T lymphocytes (identified by L3T4 antigen) were significantly lower in the exposed group. After 181 days of exposure, the total and large L3T4 lymphocyte populations were significiantly lower in exposed animals.

8. Thymus lymphocyte subpopulation quantitation.

The same quantitation in the thymus did not reveal any significant differences between the two groups.

9. Natural Killer Cell Quantitation in Spleens

Natural killer (NK) cells were identified by the surface glycosphingolipid, asialo GM_1. In general there were lower percentages of GM_1-positive cells in the exposed group; however, the differences were not statistically significant following any of the exposure periods (Table 11). There was also an increase in the percentages as the experiment progressed--controls from 22.82% to 35.51% and exposed from 21.32% to 31.71%.

D. DISCUSSION

For several years we have been interested in investigating air pollutant effects on spread and dissemination of cancer. Many studies have been carried out to identify cancer causing or carcinogenic compounds in the air we breathe, but very little attention has been given to the possibility that noxious air pollutants could influence the progression and spread of cancer without being carcinogenic. In other words, once the cancer has developed the inhalation of noxious air pollutants could enhance the progression and spread of cancer cells. We were the first to demonstrate that inhalation of ambient levels of NO_2 facilitated the spread of blood-borne cancer cells to the lungs of exposed animals (12,13). Similar findings were reported more recently from Japan by Kobayashi using ozone (31). All of these experiments suggested that the air pollutants employed had an adverse effect on the host which facilitated the spread of blood borne cancer cells. Some epidemiological studies have also indicated that the death rate for people with cancer is higher in polluted urban environments (13). This could mean increased metastasis development, since most cancer patients die from extensive cancer metastasis.

In designing animal or in vitro studies to determine air pollutant effects there is always the question of how relevant these models are to human situation. Very often only a part of the process can be investigated in one model. Such was the case when we studied the effects of NO_2 and O_3 on the spread of cancer where we investigated only the blood-borne cancer cell dissemination. Even though this was very valid information, this cancer cell metastasis model is sometimes referred to as "artificial metastasis" since many aspects of the complex process of metastasis are not represented. A model with spontaneously occurring cancer with development of metastasis would be of even greater value. This would mean that the model should have a predictable incidence of cancer and metastasis. Such animal models for solid tumors are not available. For this reason we turned to a blood cell malignancy known as T lymphoblastic lymphoma which is considered to be analogous to a lymphoma of children and young adults (23). This is an AKR mouse murine leukemia virus-associated lymphoma, where 10-15% of the animals develop the disease at the age of 1-6 months

and 71-85% at the age of 6-12 months, the mean survival being 7.6 months. It should be pointed out that a virus associated human T cell lymphoma/leukemia has recently been recognzied. In view of the foregoing, this mouse model has an additional similarity to human disease. The mouse lymphoma usually begins in the thymus gland and eventually disseminates throughout the body with extensive metastases in lungs, liver and bone marrow. By utilizing this model we were able to determine that inhalation of NO_2 affects the host and the spontaneous development and progression of this malignancy. The effect on host was measured by quantitative evaluation of major cell types in the immune system and the extent of the disease by microscopic examination of major organs of the body, following exposure of varying periods to 0.25 ppm of NO_2. Other parameters such as body weights and percent body weights for spleen and thymuses assisted in evaluating NO_2 effects. A part of this study was devoted to establish the NO_2 effect on survival of these animals. The most interesting findings came from the survival study which indicated that following the ten month period there was a significantly higher percent survival in exposed group (Fig. 1 and Table 3). The average survival of controls was consistent with reports in literature. It may appear that interpretation of results from this study may be problematic, but the multidiscipline approach, used in the study, provides enough data to present a rational interpretation of events. A partial explanation for this comes from the histopathological study which indicated that the lymphoma developed earlier in control group and showed more extensive spread following most of the exposure periods (Table 4). The latter is probably best illustrated by findings following the 37 day exposure and the last two exposure periods. The same trend, supporting earlier and more extensive lymphoma process in controls is the finding of significantly more large lymphocytes in peripheral blood during the first three exposure periods (Table 9). The large lymphocytes would be consistent with lymphoma cell morphology, while the prevalence of small lymphocytes, observed in exposed group, for the same time periods, reflect a more normal morphology (Table 8). The latter also indicates the importance of size of these cells since the total lymphocyte counts showed no differences. The peripheral blood PMN counts did not contribute to overall evaluation of the lymphoma process but were of value in confirming disseminated disease in individual cases. The most significant finding

came from the evaluation of spleen T lymphocyte subtypes. These studies represent the first attempts to investigate the effects of ambient level NO_2 (0.25 ppm) exposure on specific T lymphocyte subtypes. It is of interest that, in general, percentages of all T lymphocyte subtypes were lower in the exposed group following each exposure period. This supports our earlier studies in different mouse systems, where we observed total T lymphocyte decrease following 12 weeks of 0.35 ppm NO_2 exposure. In this study, the statistically significant differences were observed following 37 and 181 days of exposures as summarized in Table 10. The 37 day exposure which affected total spleen T lymphocytes as well as the two subtypes, seems to be of particular significance. The lymphocyte subtype designated L3T4, and known as T helper cells, plays a major role in controlling other lymphocyte functions, including proliferation, growth factor and receptor production. The advancement of lymphoma, particularly in early stages, depends upon cell proliferation or growth and the delay in advancement of lymphoma in exposed group may have been due to functional impairment of T helper cells. It is known that lymphomas during their malignant progression depend upon normal lymphocyte growth factor production. This dependency may diminish as lymphoma cells acquire a higher state of malignancy. The results from our study strongly suggest that such sequence of events may be taking place in this model system. The quantitative reduction of these cells during the first exposure period may well be linked with functional suppression, but further experiments will be needed to confirm our postulate. Moreover, the persistance of lower percentages of all T cell subtypes throughout the entire exposure period certainly indicate an adverse NO_2 effect. Statistical significance is lacking in some instances, but the possibility of biological impairment should be kept in mind, since biologically significant events are not necessarily statistically significant i.e. point mutation. Taken together the findings have indicated that the 0.25 ppm NO_2 exposure delayed fulminant lymphoma development. Thus in this study the NO_2 exposure did not enhance the advancement of malignancy, it delayed it. However, this was due to the unique nature of this malignancy which originates among the major class of cells in defense system - the lymphocytes. This delay should not be mistaken as being beneficial to the host. In fact, it is quite the opposite. The NO_2 exposure has prolonged and aggravated the disease

process by adversely affecting the quantity, and probably the function, of the cells in the immune system. It should be mentioned that similar findings have been recently reported by a group of Australian investigators who observed that in their tobacco smoke study the mouse lymphoma development was delayed in a group of smoke exposed animals. They indicated that this appears to be due to the tobacco exposed animals failing to mobilize their leukemias but persisting with a chronic lymphatic leukemia (32). They did not report any studies of T lymphocyte subtypes.

In view of the unique nature of this malignancy, that is, it develops in cells of defense system - the T lymphocytes, the findings strongly suggest that low level NO_2 exposure may affect the major subtypes of normal T lymphocytes, particularly the T helper cells. This interpretation is strongly supported by findings from the 37 day exposure since at that time most of the spleen lymphocytes should exhibit normal properties (33). This pattern of NO_2 effects agrees very well with our earlier studies of lymphocytes in another mouse strain. It is possible that growth and proliferation of lymphoma cells in the spleen is the limiting step for advancement of this disease since the thymus did not show differences in lymphocyte subtypes. To interpret the observed lower percentages of spleen Lyt2 cells (suppressor cells), as well as lack of significant quantitative differences among natural killer cells, further studies are needed. One has to keep in mind that there is a fine balance in our defense sytem between many different cells and this is the first indication that low level NO_2 induces quantitative differences among these cells. It also appears that function has been affected, but a combined study of function and quantitative changes is needed to confirm this. Such combined quantitation and functional appraisal of lymphocytes could be applied to monitor pollutant effects in human population, particularly following smog episodes. The identification of specifically affected cells and specific cell functions, instead of a function which depends upon many cell types and their interactions, would provide sensitive and more precise means for establishing hazards associated with air pollutant exposure.

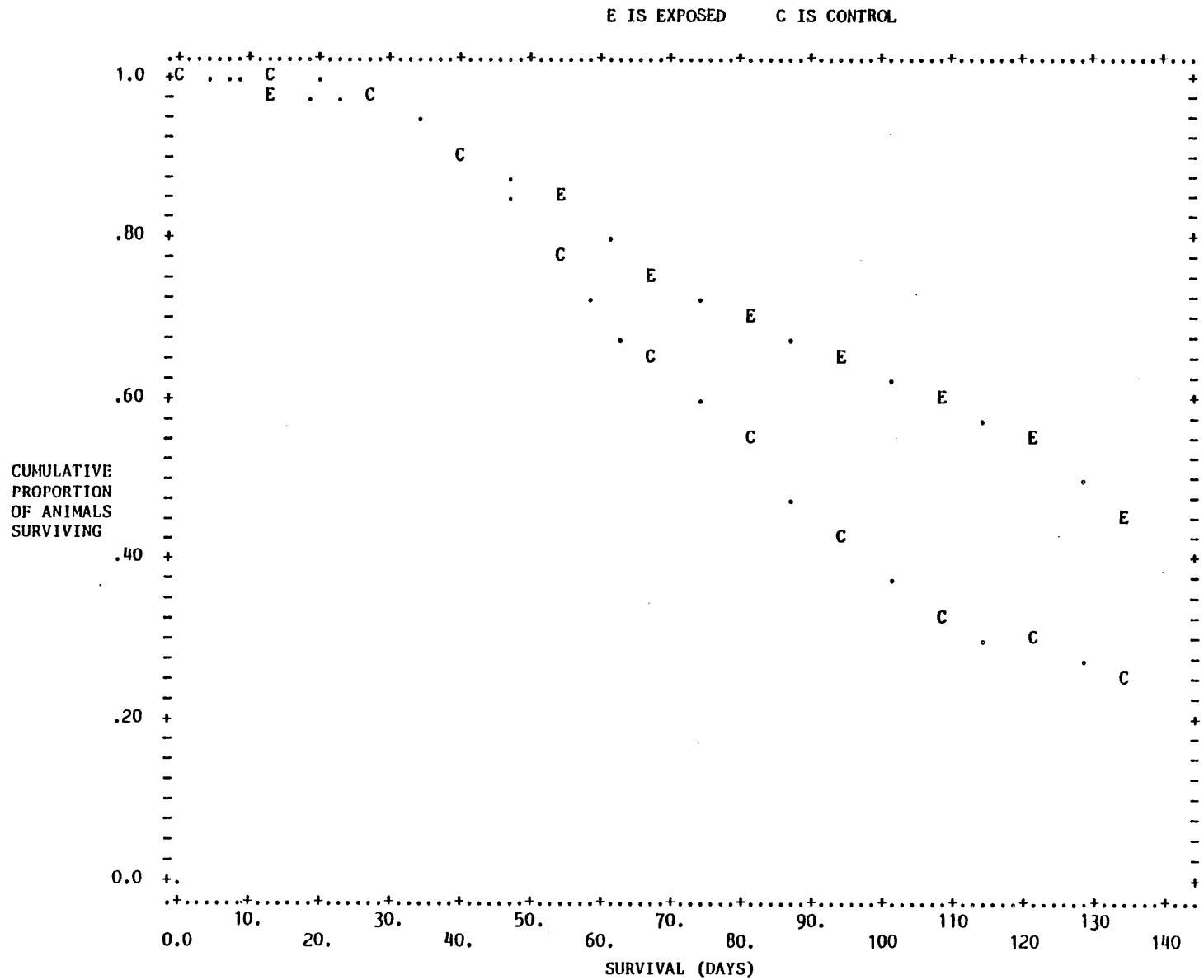

Figure 1. Survival Curve

Table 1

Percent Spleen Weight

Days of Exposure	Number of Animals Control	Exposed	Percent Spleen Wt. ± SD Control	Exposed	Significance*
37	17	18	0.402 ±0.038	0.413 ±0.050	NS
71	18	17	0.336 ±0.066	0.362 ±0.054	NS
111	18	18	0.515 ±0.572	0.562 ±0.853	NS
141	18	18	0.659 ±0.755	0.466 ±0.309	NS
181	15	18	1.137 ±1.921	0.804 ±0.866	NS

*Student's t test

Table 2

Percent Thymus Weight

Days of Exposure	Number of Animals Control	Exposed	Percent Thymus Wt. ± SD Control	Exposed	Significance*
37	6	6	0.338 ±0.083	0.340 ±0.059	NS
71	18	18	0.279 ±0.074	0.264 ±0.074	NS
111	18	18	0.424 ±0.754	0.364 ±0.518	NS
141	18	18	0.527 ±0.663	0.547 ±1.009	NS
181	15	18	0.686 ±0.978	0.571 ±0.727	NS

*Student's t test

Table 3

Survival Data Analysis

	Number of Mice			
	Total	Dead	Surviving	% Surviving
Exposed	50	25	25	50
Control	50	36	14	28
Totals	100	61	39	39

Test Statistics: $p<0.02$*

*Generalized Savage (Mantel-Cox) Statistic

Table 4

Histopathology

Days of Exposure*	Number of Animals in Experiment		Number of Animals Cat. I		Cat. II		Cat. III		Cat. IV	
	C	X	C	X	C	X	C	X	C	X
37	18	18	0	10	14	8	4	0	0	0
71	18	18	2	6	12	11	4	1	0	0
111	18	18	0	0	5	7	9	9	4	2
141	18	18	3	3	7	7	2	5	6	4
181	15	18	0	0	3	4	5	9	7	5

C - Control
X - Exposed
Cat. I - Normal
Cat. II - Early Lymphoma
Cat. III - Advanced Lymphoma
Cat. IV - Disseminated Lymphoma
*0.25 ± 0.05ppm NO_2 Exposure

Table 5

Peripheral Blood Leukocyte Counts
from 0.25ppm NO_2-exposed and Control Animals

Days of Exposure	Number of Animals Control	Number of Animals Exposed	Leukocytes per mm^3 ± SD Control	Leukocytes per mm^3 ± SD Exposed	Significance*
0	12	0	7981 ± 3869	(Baseline for 5wk old mice)	
37	17	18	11095 ± 9866	10235 ± 7592	NS
71	18	18	28252 ± 17762	28420 ± 18738	NS
111	18	18	81171 ± 72961	79881 ± 69295	NS
141	18	18	94756 ±103473	54459 ± 38072	NS
181	15	18	66252 ± 37292	81257 ± 73777	NS

*Student's t test

Table 6

Mean Percentage of PMNs in Peripheral Blood from
0.25ppm NO_2-exposed and Control Animals

Days of Exposure	Number of Animals Control	Number of Animals Exposed	Percentage of PMNs ± SD Control	Percentage of PMNs ± SD Exposed	Significance*
37	16	16	19.8 ± 5.7	19.3 ± 6.8	NS
71	18	18	30.4 ± 8.2	32.2 ± 9.2	NS
111	17	17	31.2 ±16.5	35.4 ±10.7	NS
141	16	17	35.1 ± 9.4	32.1 ± 7.6	NS
181	15	18	44.1 ±10.7	41.2 ±15.7	NS

*Student's t test

Table 7

Mean Percentage of Lymphocytes in Peripheral Blood from 0.25ppm NO_2-exposed and Control Animals

Days of Exposure	Number of Animals Control	Number of Animals Exposed	Mean % of Lymphocytes ± SD Control	Mean % of Lymphocytes ± SD Exposed	Significance*
37	16	16	79.7 ± 5.7	79.9 ± 8.4	NS
71	18	18	66.1 ±11.4	65.7 ± 9.6	NS
111	17	17	68.8 ±16.4	62.1 ±12.1	NS
141	16	17	64.8 ± 9.5	67.9 ± 7.5	NS
181	15	18	55.6 ±10.7	55.7 ±18.3	NS

*Student's t test

Table 8

Mean Percentage of Small Lymphocytes in Peripheral Blood from 0.25ppm NO_2-exposed and Control Animals

Days of Exposure	Number of Animals Control	Number of Animals Exposed	Mean % of Lymphocytes ± SD Control	Mean % of Lymphocytes ± SD Exposed	Significance*
37	16	16	38.5 ±11.6	69.9 ±10.5	p==>0
71	18	18	23.1 ± 7.5	33.7 ±14.5	p<0.01
111	17	17	9.4 ± 9.3	27.7 ±15.1	p==>0
141	16	17	10.2 ± 6.6	15.1 ±12.3	NS
181	15	18	15.3 ± 6.8	14.7 ± 7.6	NS

*Student's t test

Table 9

Mean Percentage of Large Lymphocytes in Peripheral Blood from 0.25ppm NO_2-exposed and Control Animals

Days of Exposure	Number of Animals Control	Number of Animals Exposed	Mean % of Lymphocytes ± SD Control	Mean % of Lymphocytes ± SD Exposed	Significance*
37	16	16	41.3 ± 9.7	14.9 ±10.2	p==>0
71	18	18	42.9 ±12.1	32.0 ±13.7	p<0.02
111	17	17	59.4 ±17.4	34.4 ±10.9	p==>0
141	16	17	54.8 ±14.0	51.7 ±17.3	NS
181	15	18	40.6 ±13.4	41.1 ±20.1	NS

*Student's t test

Table 10

Spleen Lymphocyte Subtype Percentages in Control and 0.25ppm NO_2-Exposed Mice

Lymphocyte Subtype*	Lymphocyte Population	Days of Exposure	Number of Animals Control	Number of Animals Exposed	Subtype Percentage Control	Subtype Percentage Exposed	Significance** p
Lyt-2	Large	37	18	18	15.8 ± 3.6	13.3 ± 2.5	0.037
Thy-1.2	Total	37	18	18	39.2 ± 4.1	36.4 ± 4.8	0.034
L3T4	Small	37	18	18	28.0 ± 2.8	25.7 ± 3.1	0.019
L3T4	Total	181	18	18	40.4 ±19.4	26.8 ± 6.5	0.003
L3T4	Large	181	15	18	59.9 ±12.8	45.7 ±12.2	0.007

*Thy-1.2 - Total T cells
Lyt-2 - Cytotoxic/Suppressor T cells
L3T4 - Helper/Inducer T cells

**Mann-Whitney U-Test

Table 11

Percentage of Spleen Natural Killer Cells (asialo GM_1-positive) in Control and 0.25ppm NO_2-exposed Animals

Days of Exposure	Number of Animals Control	Number of Animals Exposed	Mean % of Spleen Cells ± SD Control	Mean % of Spleen Cells ± SD Exposed	Significance*
37	18	18	22.8 ± 4.2	21.3 ± 4.0	NS
71	18	18	30.9 ±13.6	27.4 ±10.9	NS
111	18	18	35.9 ±17.1	31.4 ±13.7	NS
141	18	18	31.6 ±14.2	29.7 ± 8.8	NS
181	15	18	35.5 ±11.5	31.7 ± 9.0	NS

*Mann-Whitney U-Test

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12. PUBLICATIONS

During this contract period, utilizing data from this and some of the previous contracts, we have put together the following publications:

1. Richters, A., Damji, K. and Richters, V. Immunotoxicity of nitrogen dioxide. J. Leuk. Biology 42:413-414, 1987.

2. Kuraitis, K.V. and Richters, A. Spleen cellularity shifts from the inhalation of 0.25-0.35 ppm nitrogen dioxide. J. Environ. Path. Toxicol. Oncology (in press).

3. Richters, A. and Richters, V. Nitrogen dioxide (NO_2) inhalation, formation of microthrombi in lungs and cancer metastasis. J. Environ. Path. Toxicol. Oncology (in press).

4. Richters, A. Effects on nitrogen dioxide and ozone on blood-borne cancer cell metastasis. J. Toxicol. Environ. Health (submitted).

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