Response of a Virus-induced Leukemia in Mice to High Oxygen Tension*

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SUMMARY

Effects of repeated short treatments with high pO₂ (oxygen partial pressure), at about 2.5 atm. and administered over a relatively long period, on the course of a leukemia induced by Friend virus were investigated. Treatment with high pO₂ during the first 3 or 6 weeks, or even during the second 3 weeks of the disease, reduced the intensity of leukemic symptoms markedly below those in the untreated inoculated group, but the effect was not permanent.

It is argued that the beneficial effects of high pO₂ treatment should be regarded at least in part as being due to a specific availability of higher than normal tissue pO₂'s, rather than simply to the relief of hypoxia inside tumorous masses.

Several lines of evidence have pointed to the desirability of a renewed investigation of the possible therapeutic effects of high pO₂ (oxygen partial pressure) treatments on malignant disorders.

a) The known toxic effect of high pO₂'s on living cells and on a number of specific metabolic reactions (e.g., 17) may affect actively growing and multiplying cells, more sensitive than normal mature tissues. Indeed, even moderate increases in O₂ tension above 0.2 atm. have been reported to reduce markedly the growth rate of normal cells in a suspended culture (6) and similar inhibitory effects have been found on the rate of multiplication in a culture of HeLa strain carcinoma at pO₂'s above 0.3 atm. (3, 16).

b) Higher pO₂'s increase the sensitivity of normal and neoplastic tissues to damage by x-radiation and have been coupled with x-ray therapy (e.g., Gray [10]). Evidence has been developed which supports the postulate that the damaging effect of either oxygen poisoning or irradiation is due to a common action—perhaps the production of oxidizing free radicals (2, 9).

c) Some years ago Warburg pointed out (e.g., 19) that neoplastic tissues have a high rate of anaerobic and aerobic glycolysis and suggested that this metabolic source of energy may be crucially important to the maintenance of growth and multiplication in such cells, in the presence of what he regarded to be an inadequate respiration. The rate of glycolysis in tissues, even neoplastic ones, tends to be reduced as the oxygen tension is raised from 0 to atmospheric levels—i.e., to 0.2 atm. (the Pasteur effect). If the high aerobic glycolysis rates of neoplastic cells were to be inhibited further by still higher pO₂'s (but see 1, 12, 16, 18), one would expect, on Warburg's hypothesis, some suppression of neoplastic growth.

A number of tests of high pO₂ therapy on a variety of animal tumors have been conducted by a number of investigators, but with variable and apparently inconclusive results (17). A common feature of these earlier tests, however, was their use of only one or a few treatments of the animals with the high pO₂, with a treatment duration of some hours, or until death. In some instances only modest pO₂'s of about 1 atm. were used. A long-lasting single exposure, however, might tend to reduce the selectivity of any depressant effect of high pO₂ on neoplastic activity, as the more general effects of O₂ poisoning may supervene earlier. Therefore, it seemed to us that a more adequate test of the potentialities of high pO₂ should utilize repeated short applications over longer total periods of time.
It has been proposed by Gray (10) that the increase in radiosensitivity of tumors produced by breathing higher than normal pO2's occurs primarily in the hypoxic portions of the tumors which still retain reproductive integrity. Tumor cells appeared to show little further gain in radiosensitivity if their ambient pO2 was increased above ordinary aerobic levels—i.e., above 40 mm. Hg. The special usefulness of breathing high pO2 according to this view lies in its bringing the pO2 within anoxic tumor masses up to more normal aerobic levels.

The present experiments thus aimed to test two points simultaneously: (a) Do high pO2's alone, when given in short exposures repeatedly and over a relatively long total period, effectively antagonize the progress of a neoplastic disorder? (b) Can any such effect of high pO2's be demonstrated in a neoplastic disorder which does not involve the appearance of large, relatively poorly vascularized tumorous masses—i.e., one in which there is presumably no problem of hypoxia in some portion of the neoplastic tissue? In their study of the pathology of the leukemia induced in mice by the Friend virus, which was used in the present study, Metcalf, Furth, and Buffett (13) found no evidence of massive tumor-like accumulation of cells, except in a few rare instances.

MATERIALS AND METHODS

Virus material and mice.—Four- to 6-week-old, albino female mice, weighing 15-20 gm. and of Swiss strain (supplied by Simonson), were used in the main experiment. The body weights increased to 22-30 gm. by the end of the 3d week, and to 30-35 gm. by the 12th week. In a large preliminary experiment similar mice, but supplied from the Hooper Institute breeding colony, were used. The virus was obtained by making a 20 per cent homogenized suspension of enlarged spleens from mice infected with the Friend virus. The suspension of spleens was clarified by centrifugation at 2500 r.p.m. for 20 min., and 0.5 ml. of this fluid was injected intraperitoneally into each animal. The Friend agent was obtained originally from the Virus Laboratory, University of California, Berkeley, and had been subsequently serially passaged some dozen times by intraperitoneal inoculations of clarified spleen suspensions.

Oxygen treatment.—In order to maximize any effects of high pO2, the aim was to subject the mice to as high a pO2 as they could tolerate without dying of oxygen poisoning. The levels used were thus sufficient to produce symptoms of distress (abnormal breathing, manic activity, and, less commonly, lying down and convulsive movements) in some of the mice in each group, in some though not all of the treatments. Some difference in the tolerance of high pO2's, as administered here (see below), was encountered among different batches of mice. The batch of mice in the preliminary experiment, Swiss albinos, Hooper Institute-bred, tolerated a pO2 of 2.7 atm. (i.e., pure O2 at 25 p.s.i. above atmospheric pressure) for the period used. When the main experimental group (Swiss albino, Simonson) was started at a pO2 of 2.7 atm., several O2 poisoning deaths occurred. The pressure was dropped to 2.5 atm. the next day. However, since excessive difficulties among the mice continued, after 1 week the treatment pO2 was lowered to 2.25 atm. for the remainder of the main experiment. The dead mice were partially replaced by others from the same stock batch of inoculated animals.

The pressure chamber was a rectangular box (20 X 28 cm. and 15 cm. high, inside; volume approx. 8.4 l.) of brass, but with a removable thick lucite top which permitted observation of the animals during treatment. The size of the chamber unfortunately limited the number of mice to 25-30 in each treatment. Higher temperatures appeared to increase the exhibition of symptoms of severe O2 poisoning in the mice (see also Campbell [4]). Consequently, the chamber was partially immersed in a cold water bath which maintained the temperature inside the chamber at about 21° C. ± about 1° C. This resulted in some condensation of moisture on the inner surface of the chamber's walls. To prevent the mice from wetting their fur, large mesh wire screening was installed as a lining of the chamber in such a way as to maintain a small space between the mice and all the inner surfaces except for the top.

With the mice enclosed, the air in the chamber was flushed out with pure O2 at atmospheric pressure for 1 or 2 min. Then total oxygen pressure was gradually raised to the desired maximum over a period of 15 min., maintained at the maximum for 30 min., and lowered to atmospheric level for another 15 min. The mice were removed to their cages immediately. During the entire period of oxygenation a ventilating O2 flow of 3-5 liters/min was maintained, the gas exiting via a small controlled leak in the outlet. Each group of mice undergoing O2 treatment was given this experience once in the morning and once in the afternoon on each of the 5 weekdays, Saturdays and Sundays being omitted.

Design and analysis of experiments.—The course of the disease was assessed, following Metcalf et al. (19), by measurement of spleen weights and of lymphocyte and erythroblast counts in the blood.
This was done at the end of the 3-, 6-, and 12-week periods following inoculation with the virus. Spontaneous deaths associated with enlarged spleens were counted as leukemic fatalities. Since such deaths usually occurred during nonworking hours, proper blood counts are not available for these animals; also, their spleen weights were not included in calculation of the mean value given in Chart 1, but are discussed for comparison in the text. Blood samples were obtained by snipping the tail. The relative proportion of lymphocytes and erythroblasts among all nucleated cells were obtained from differential counts of smears stained with Wright's stain. The actual count per cu. mm. of each cell type was calculated from the total count of nucleated cells, which was obtained after dilution in the standard way.

In the main experiment 109 mice were given inoculations of the virus, 30 additional randomly selected mice from the same batch serving as a normal group (N). One hundred of the inoculated mice were immediately divided at random into four main groups, and each of these into appropriate subgroups (the extra seven were distributed into these groups 2 days later to make up losses from O₂ poisoning). Group I (36 mice given inoculations) was the virus group untreated with O₂. Group II (31 mice given inoculations after some initial O₂ deaths) was treated with high pO₂ during the first 3 weeks, starting on the day after inoculation. Group III (twelve mice given inoculations after some initial O₂ deaths) was treated with high pO₂ during the first 6 weeks, starting on the day after inoculation. Group IV (fifteen mice given inoculations) was treated with high pO₂ after the first 3 weeks, starting on the day after inoculation. Group I (36 mice given inoculations) was the virus group untreated with O₂. Group II (31 mice given inoculations after some initial O₂ deaths) was treated with high pO₂ during the first 3 weeks, starting on the day after inoculation. Group III (twelve mice given inoculations after some initial O₂ deaths) was treated with high pO₂ during the first 6 weeks, starting on the day after inoculation. Group IV (fifteen mice given inoculations) was treated with high pO₂ after the first 3 weeks, starting on the day after inoculation. Group I (36 mice given inoculations) was the virus group untreated with O₂. Group II (31 mice given inoculations after some initial O₂ deaths) was treated with high pO₂ during the first 3 weeks, starting on the day after inoculation. Group III (twelve mice given inoculations after some initial O₂ deaths) was treated with high pO₂ during the first 6 weeks, starting on the day after inoculation. Group IV (fifteen mice given inoculations) was treated with high pO₂ after the first 3 weeks, starting on the day after inoculation.

In the initial preliminary experiment it was found that the pO₂ levels used here have a negligible effect on the spleen weights and cell counts in normal animals.

All mice surviving at the end of the 12-week period were sacrificed and their spleen weights and blood counts taken. To obtain some measurements of spleen weight at the earlier times during the experiment, approximately ten mice were sacrificed in each of Groups I and II after the first 3 weeks, and approximately five mice were sacrificed in each of Groups I, II, III, and IV after the first 6 weeks. Blood counts were made on these sacrificed mice as well as on a large fraction of the unsacrificed mice. Note that, at the end of the first 3 weeks, Group III has had the same experiences as Group II, and Group IV the same as Group I; Groups III and IV were therefore not themselves examined for spleen sizes or blood counts after the first 3 weeks. In a preliminary experiment the mice to be sacrificed at the end of the 3- and 6-week periods were taken from the appropriate whole group that contained them. Even though the attempt was made to pick these out at random, there was evidence that in fact the sicker mice tended to be chosen by this procedure, leaving behind the mice with the milder symptoms of the disease to survive to the end of the experiment. Consequently, in the main experiment each group of mice given inoculations was immediately subdivided randomly into sub-groups containing the appropriate number to be sacrificed at the end of 3 weeks, 6 weeks, and 12 weeks, respectively, no interchange among these subgroups being subsequently permitted. Since blood counts were not made on all the unsacrificed mice at the 3- and 6-week times, they were made on a whole subgroup (in one cage) of the unsacrificed mice, for the same reason just given above.

**RESULTS**

The results of the main experiment are summarized in Chart 1. It may be seen that, at the end of 3 weeks, high pO₂ Group II had mean spleen weights and erythroblast counts about 50 per cent of those in the untreated, inoculated Group I; lymphocyte counts were not greatly different, but there was only a mild increase in this value in Group I at this time. By the end of 6 weeks, O₂-treated mice in Groups II and III were even more strikingly different from group I; e.g., the raised mean lymphocyte count (above the normal mean value in Group N) was approximately 6000 for the former groups to 23,000 for the latter, and the ratio of erythroblast counts is even smaller than this—1900 to 9500. Since mean values can be heavily weighted by a few greatly abnormal values, it is also instructive to look at the distribution of individual points in Chart 1. Most of the individual values for the mice in the treated groups fall well below most of those in the untreated, inoculated Group I at the 3-week and 6-week times (except for the 3-week lymphocyte counts, which had not yet become severely abnormal in Group I). It may also be added that, although the number of values of spleen weights at the 6-week time is relatively small, the distribution of value around each mean is unique to each group.

It may also be seen that the mice treated with O₂ during the first 3 weeks only (Group II) appeared to be as much benefited at the end of the 6th week as those which had received O₂ therapy during the entire 6 weeks (Group III). It would thus seem that the first 3 weeks' treatment continued to exert an influence on the development of
CHART 1.—The effects of high pO₂ treatments on the individual spleen weights (A), erythroblast counts (B), and lymphocyte counts (C) in Friend virus leukemia.

In each graph all the measurements available in the main experiment are plotted (except for the unsacrificed mice that died spontaneously of leukemia). In order to keep the points for the various groups of mice intelligibly distinct on the graph, a separate vertical column is maintained for each group at each harvest time. That is, zero points for the time base are placed at successive points to the right, starting from the origin, for each succeeding group number (as noted in C). The vertical columns in each set are plotted in the following order of groups (all the groups are represented at the 12-week harvest time): Group N (•) normal, uninoculated mice; Group I (O) mice inoculated with virus only; Group II (x) inoculated and treated with high pO₂ during the first 8 weeks only; Group III (A) inoculated and treated with high pO₂ during the first 6 weeks; Group IV (L) inoculated and treated with high pO₂ during the second 3 weeks only. The same arrangement is carried through for those groups that are represented in the 3-week and 6-week harvest sets. Values for Groups III and IV start only at the 6th-week harvest time, but they should be similar, for the first 3 weeks, to Groups II and I, respectively.

A line connects the mean values of the vertical columns for a given group. The very large values for lymphocyte count, 415,000, and erythroblast count, 50,000, in one mouse of Group I at the 12-week time are not included in the mean values of that set of eleven mice. If they are included, the mean values become 53,800 instead of 17,600 for lymphocytes and 6700 instead of 2400 for erythroblasts. Points representing the spleen weights and time of death in the mice that died of the leukemia before their harvest-sacrifice time are not included in part A. The spleen weights in the spontaneous leukemic fatalities (almost all of which occurred during the last 6 weeks, see text) were well above 1 gm. in every case (except one at 0.9 gm.).
symptoms well beyond this period. On the other hand, the effects of the treatment were evidently not permanent. All the groups given high pO2 treatments at some time during the first 6 weeks but not thereafter, at the end of 12 weeks showed spleen weights and cell counts that were not significantly different from untreated Group I. The somewhat higher spleen weight values in some of the O2-treated groups, as compared with untreated Group I, at the 12-week time, become less different if the spleen weight of the spontaneous fatalities during the second 6-week period are included in the mean values. The values then become 1.21 gm. (twenty mice), 1.11 gm. (sixteen mice), 1.42 gm. (seven mice) and 1.41 gm. (nine mice), for Groups I, II, III, and IV, respectively. Blood counts are not available for the mice that died of the leukemia, including deaths in the last day’s handling before being sacrificed, but the counts would be expected to be very high in these mice.

The values in Group IV, which was treated only during the 4th, 5th, and 6th weeks, were close to, though not quite as favorable as those for Groups II and III. It seems clear, then, that high pO2 treatments could suppress symptoms that had already developed—i.e., they brought about a large reduction in spleen size and erythroblast count, while markedly holding down the increase in lymphocyte count that occurred in the untreated, diseased mice.

The individual values show that virtually 100 per cent of the inoculated mice, even the O2-treated ones, showed some degree of the leukemic symptoms (some enlargement of spleen and some erythroblastosis; lymphocyte values showed more overlap with the normal mice, at the 8d- and 8th-week times). At 12 weeks the incidence of values in the near normal range were not especially different, percentage-wise, among all the inoculated groups. Thus, it appears that, when the high pO2 treatments were effective, they did not abolish the disease in any of the individual mice; rather, they appeared to suppress the intensity of the symptoms in a large percentage of the mice.

The influence of high pO2 treatment on mortality rate was less clear. During the first 3-week period, there were two leukemic deaths out of 51 mice in Groups I and IV (virus only during this time), and none in the remaining groups. During the second 3-week period there were no leukemic deaths in any of the groups. During the second 6-week period, there were five leukemic deaths out of twenty remaining mice (25 per cent) in Group I and seven out of 35 (20 per cent) for Groups II–IV combined. In addition, a number of the mice which had been given inoculations (no normal mice) died before blood could be drawn during the relatively mild handling involved in obtaining a blood sample on the last day of the experiment. These mice had large spleens. If such deaths are regarded as due to the leukemia, and are included in the “spontaneous” leukemia mortality figures, the rates for the second 6-week period become 45 per cent for Group I, and 25 per cent for Groups II–IV combined. Since the experiment was terminated after 12 weeks, the total potential mortality rate in any of the groups in the present experiment is not known. If the last day deaths are counted, and early deaths in Group I also, the mortality rate for the first 12 weeks in the O2-treated mice becomes approximately half of that of the untreated mice for this 12-week period.

The results of a large initial, preliminary experiment were qualitatively similar to those described above for the main experiment, for those portions of the initial experiment in which the procedural factors were rather similar. In the preliminary experiment an additional group of normal (not inoculated) mice was treated only with high pO2. This group showed no appreciable differences from the normal unoxygogenated mice, except for their showing some erythroblasts (but only about 100 per cu. mm.) during the period of O2 treatments.

**DISCUSSION**

The results appear to demonstrate that treatment with high pO2 can suppress the development of the leukemic symptoms or can cause them to regress after they are developed, although these effects were not permanent after the O2 treatments were stopped. There was some indication of a reduced mortality rate also, but this result was not clear-cut and should be investigated further. There is no information in the present experiments as to whether continual treatments with high pO2 beyond the initial 6-week period would have continuously suppressed the symptoms and mortality rate; but the finding that O2 treatments begun after the disease had developed were still at least partly effective indicated that this point should be pursued further. Prolonged breathing of oxygen at a level (0.7 atm.) lower than that tested here was reported to be without effect on a spontaneous leukemia (7). In the present work, the return of the more severe symptoms by the 12th week, after the cessation of O2 treatments at the end of the 6th week, parallels the finding that cell multiplication in cultures was resumed at the undiminished rate, if the suppressor pO2 levels were reduced sufficiently soon to below 0.2 atm. (3).
Since there is no evidence of tumorous masses in the leukemic disorder being tested (12), it may be assumed that the abnormal tissues in the Friend virus leukemia have a normal supply of oxygen. It should therefore be concluded, at least tentatively, that a higher than normal aerobic tissue pO₂ is exerting suppressor effects on the neoplastic process here. This correlates with the findings that pO₂ in a cell culture must exceed 0.2 to 0.3 atm. to become markedly inhibitory to the multiplication rate (3). It would follow that the important factor is not an improvement in the utilization of O₂ by the tissues but rather the specific effect of a certain pO₂ itself. Oxygen tensions above the normal aerobic levels, if anything, decrease respiratory rate rather than increase it (8, 18).

It is conceivable that the high pO₂ acted directly on the virus molecule or on its interaction with the host cells, rather than on the "neoplastic reactions." This problem has been pointed out by Chirigos et al. (5) to be one of those common to tests made with virus-induced neoplasms. The possible direct effects of high pO₂ on the virus molecule itself, however, may merit investigation, especially in view of the report (15) that high pO₂ treatments afforded some protection against certain neurotropic virus infections in mice.

It should be emphasized that high pO₂ treatment, used without any adjuvants, was the effective agent in the present experiments. Thus, the action of high pO₂'s in increasing the sensitivity to irradiation need not be regarded as a specific adjuvant to irradiation, but rather as a general effect to be expected because of the O₂ action itself (see also Gray [10, 11]). One is therefore encouraged to propose that high pO₂ or an agent that simulates it may be a useful synergist which might be added to any other effective treatment of neoplastic disorders.

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1 The enlarged spleen itself might be thought of as a large tumorous mass, but there is no reason to think that its tissues have an impaired circulation. Splenectomy, however, does prevent the erythroblastosis and markedly delays the appearance of a leukocytosis in mice infected with the Friend virus (13, 14). The suppressing action of high pO₂ on spleen size, as well as on the erythroblastosis and lymphocytosis, is consistent with the view that it exerted its effects on the primary site of the neoplastic process.

REFERENCES

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