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The Effect of Hyperbaric Oxygen Treatment on Pulmonary Metastasis in the C3H Mouse*

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Abstract—*The incidence of pulmonary metastases in mice exposed to HBO treatment was investigated in two systems: artificial exogenous metastasis to the lung after i.v. inoculation of tumour cells and the endogenous metastatic spread from spontaneous tumours. Exposure to HBO decreased the lung colony count after inoculation with ELD cells, but had no effect on lung colonisation by C3H mammary tumour cell aggregates. Lung metastasis increased in mice with spontaneous tumours exposed to HBO. This is believed to be a result, not of transient lung damage associated with HBO, but of increased dissemination of cells from the primary tumour.*

INTRODUCTION

THE VALUE of hyperbaric oxygen (HBO) treatment in radiotherapy is still being assessed [1, 2]. One aspect of this form of treatment that has to be considered is any increased morbidity that may result. In particular, it has been suggested that HBO treatment can be correlated with an increased incidence of distant metastatic deposits [3]. Evidence from the earlier clinical trials is equivocal and more recent reports are conflicting: some centres report an increased incidence of metastasis [2, 4] while others do not [5]. The picture is equally confused when animal experiments are considered: a decreased incidence of lung metastatic deposits in mice injected with allogeneic tumour cells has been claimed [6], whilst work with a syngeneic tumour did not substantiate this [7].

We had noted that after HBO tank exposure using C3H/Bts female mice with spontaneously arising mammary tumours the mice subjected to HBO treatment had a higher incidence of lung metastatic deposits than was usually encountered in this strain. The following experiments were designed to investigate this finding further.

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Two complementary series of experiments were carried out: the exogenous technique, where mammary tumour cells are injected i.v. into syngeneic recipients and the resulting lung nodules assayed, and the endogenous, where the incidence and number of blood-borne lung metastases are compared in matched groups of C3H/Bts mice bearing spontaneous, not transplanted, mammary tumours. The exogenous lung colony assay technique was also carried out using a line of diploid ascites (ELD) cells.

MATERIALS AND METHODS

Mice

All mice used were from the C3H/Bts colony. These mice are conventionally maintained, caged eight to a box and fed Dixon's CDDM cubed diet with water *ad libitum*. Female mice bearing spontaneous mammary tumours were used either as tumour cell donors or in the endogenous experiments: 15-week old male mice were used as recipients for the lung colony assays.

HBO exposure

Mice were subjected to HBO in a small animal chamber manufactured by Vickers Ltd. [8]. The exposure schedule was the same

as that used for the treatment of patients in the Radiotherapy Department of St. Bartholomew's Hospital, i.e., 6 exposures in 18 days. Eight mice were placed in the chamber at one time, and the chamber flushed out with 100% oxygen at 2 ATA (103 kPa) for 5 min, with a flow rate of 4–5 l/min. Pressure was raised to 3 ATA (207 kPa) over a period of 20 min and maintained at 3 ATA for a further 20 min. Decompression was effected over a 5 min period. All mice were unanaesthetised during HBO treatment. Control animals were placed in the chamber, unpressurized, for 30 min.

Lung colony assay

For lung colony assays mice were injected via the tail vein with a suspension of tumour cells prepared by trypsinising the solid mammary tumour [9]. Animals were injected with tumour cells either the day before or the day after a course of 6 HBO exposures. An additional tumour, a transplanted, allogeneic Ehrlich Landschutz Diploid (ELD) tumour, was also investigated using this technique [10]. This tumour grows as an ascites tumour and cells were diluted in medium and injected without need for trypsinisation. Cell suspensions of both tumours were suspended in Eagle's medium plus 10% foetal calf serum prior to injection. As previous work in this laboratory has shown that single cell suspensions of the C3H mouse mammary tumour do not form lung colonies, it was necessary to inject a suspension containing cell aggregates of this tumour. A linear relationship exists between the number of aggregates injected and the resultant number of lung colonies [9]. Animals were killed either 2 weeks (ELD cells) or 6 weeks (mammary tumour cells) post-injection and their lungs were examined for peripheral tumour colonies after inflation with Bouin's fixative.

Spontaneous tumour-bearing mice.

Female mice developing mammary tumours are withdrawn from the stock colony and recaged as described above. The mean age of detection of the first tumour in these experiments was 14.5 months (range 8–22 months).

Three orthogonal tumour diameters are measured three times a week over the initial period of tumour growth, and the product of these diameters is used as an index of tumour volume. The volume doubling time of the tumour can then be calculated. Since volume doubling times of spontaneous mouse mammary tumours vary so widely [11], as opposed to the doubling times of first generation transplanted tumours, groups for experiment are made up of mice with tumours matched for initial tumour volume and volume doubling times. HBO treatment was given to 22 mice whose tumours had a mean volume doubling time ($\pm$S.E.) of 20.2 ± 2.8 days (range 8.1–38.5 days) and 22 control mice had tumours with a mean volume doubling time ($\pm$S.E.) of 16.2 ± 2.3 (range 8.5–44.3 days). Mice were weighed and their tumours measured during the initial pre-treatment period, HBO treatment and until the end of the experiment. Mice were killed *in extremis*, or when the tumour was so large as to be causing marked discomfort. All mice were examined *post mortem*. The incidence and number of lung nodules was noted after fixation in neutral buffered formol solution, and the left lung lobe and each of the 4 right lung lobes cut into 1.5 mm slices for histological examination. One 5 μm section was examined from each block. No lung nodule was scored as positive without histological confirmation of the presence of mammary tumour cells in the lungs. A sagittal section of the brain was examined, after the brain had been fixed inside the opened cranium. All the mammary tumours of mice in this experiment were assessed as adenocarcinoma of a simple acinar type (Dunn's Type A) [12].

Lung damage

An attempt has been made to evaluate the extent of pulmonary damage in these mice by scoring slides blind using an arbitrary list of 8 pathological changes, chosen to cover both the acute changes that might be seen soon after HBO and the chronic changes typical of old conventionally maintained mice. These changes were: haemorrhage into alveoli, thickened alveolar septae, pulmonary congestion, consolidation, presence of alveolar macrophages, lymphocyte cuffing of bronchioles and vessels, emphysema, and collapse. Each category was graded as mild or severe. Scores from right and left lobes were summed and halved, giving a maximum damage of 16.

RESULTS

Effect of HBO treatment on non-anaesthetised mice

During the first of the 6 compression treatments, all the mice were excited and moved constantly round and round the pressure chamber, scrambling over each other. This

behaviour became less marked during subsequent exposures and for the last two pressurisations the mice sat quietly in the tank and groomed themselves. All the young male (tumour-cell recipient) mice survived this procedure and showed no ill effects. The older tumour-bearing female mice tolerated HBO exposure less well. Four mice became distressed within 48 hr of treatment (one after the 2nd exposure, one after the 4th and two after the 5th) and were killed. *Post mortem* examination showed severe lung damage with oedematous alveolar walls and massive haemorrhage into the alveoli. No brain damage was found.

Lung colony assay

Table 1 shows the lung colony assay results from mice injected with ELD cells. Mice injected with cells prior to HBO exposure had a significantly reduced number of colonies ($0.02 < P < 0.05$).

Table 1. *Lung colony assay—ELD cells* (10^6 *cells injected*)

Time of injection relative to HBO exposures	Mean number of lung colonies (±S.E.)
18 hr before	10.4 ±2.5
18 hr after	25.6 ±5.9
Control	24.25±2.4

Table 2 shows the results for mice injected with mammary tumour cells. There was no significant difference ($P > 0.05$) between the number of lung colonies found in mice injected before, after or without HBO exposure.

Table 2. *Lung colony assay—mammary tumour cells* (1×10^5 *aggregates rejected*)

Time of injection relative to HBO exposures	Mean number of lung colonies (±S.E.)
18 hr before	128.8±21.8
18 hr after	141±21.3
Control	147±16.8

Mice with spontaneous mammary tumours

Survival. Omitting the 4 mice killed with 48 hr of HBO exposure, mice surviving all 6 exposures lived for the same length of time (mean ± S.E. = 80.2 ± 10.1 days) after treatment as the untreated controls (69.1 ± 7.6 days).

Effect on lung metastatic deposits. The incidence of lung metastatic deposits in the HBO treated mice was greater than that of the non-pressurised controls—66.6% of the treated mice had lung nodules on gross inspection as compared with 36.0% of the control mice (χ^2 test—significant at the 5% level).

The incidence of metastasis scored on a histological basis is higher than the figure obtained from macroscopic inspection, since the presence of arterial emboli of tumour cells is scored as positive. The incidence of metastasis is 88.8% in the HBO treated mice and 65% in the control mice.

The mean number of lung colonies per mouse in the lungs of mice with metastases was the same for both groups—10.4 ± 0.2 and 9.9 ± 0.3 for HBO treated and control groups respectively.

Effect of HBO treatment on primary tumour growth

The growth rate of a solid tumour as judged from a semilog plot of volume against time is approximately exponential over the early life of the tumour. In the case of the slowly growing spontaneous tumour used here, an exponential fit is obtained until the tumour "volume" exceeds 3000–4000 mm^3, and in many cases the growth curve is exponential until 6000 mm^3 is reached. There was no evidence that 6 exposures to HBO altered the rate of growth of the tumours in any way. As an illustration of this, Fig. 1 shows that the same straight line may be fitted to both pre-treatment and post-treatment points. This mouse developed two subsequent mammary tumours, which appeared during HBO treatment, and these also followed a similar growth pattern.

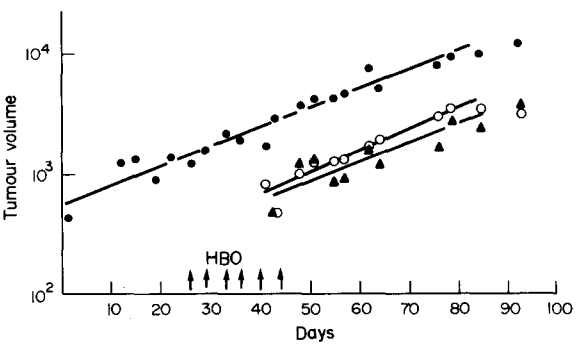

Fig. 1. *Growth rates of first and subsequent spontaneous mammary tumours during* 6 *exposures to HBO. First tumour (closed circles) volume doubling time*, 19.2 ± 1.3 days, r = 0.96. *Second tumour (open circles) volume doubling time* 18.3 ± 1.7 days, r = 0.96. *Third tumour (closed triangles) volume doubling time* 19.0 ± 3.4 days, r = 0.89.

The development of second and subsequent primary tumours is associated with a slightly increased tendency to pulmonary metastases [13] and in experiments of this type it is necessary to check that the distribution of multiple tumours is the same in both treated and untreated groups. Table 3 shows that this was the case, so the increased incidence of metastases in the HBO treated mice was not influenced by multiple tumour formation.

Table 3. Development of multiple tumours

No. of mammary tumours	No. of HBO treated mice (18)	No. of untreated mice (22)
1	11 (61.1)*	14 (63.6)
2	5 (27.7)	5 (22.7)
3	2 (11.1)	3 (13.6)

*Figures in parentheses are percentages.

Lung damage

The extreme pulmonary damage seen in the 4 mice killed during HBO treatment was not found in the 18 mice who survived the treatment. Figure 2 shows the distribution of

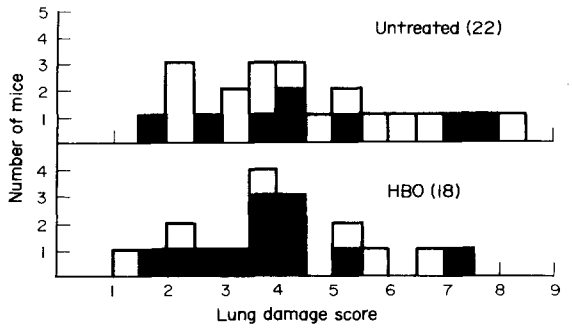

Fig. 2. Pulmonary metastatic deposits related to damage scored. □ = no metastases. ■ = gross metastases.

damage scored at *post mortem* examination in both untreated and HBO treated mice. There was little difference between the two groups; the mean score (±S.E.) was 4.3±0.35 for untreated and 4.6±0.41 for HBO mice. Even with these small groups it is clear that mice surviving HBO treatment have no greater residual damage 2–3 months afterwards than untreated control animals. Equally the distribution of metastases follows the same general pattern: metastatic deposits are not concentrated in those mice showing the most pathological changes in the lung, i.e., to the right of the histogram.

Table 4 summarises the degree of damage under individual headings, rather than the total damage scored. Again there is no overall difference between the untreated and the HBO treated groups (lines 1 and 4). If the groups are broken down further (lines 2 and 5, 3 and 6) to correlate different types of lung damage scored between mice with and without metastatic deposits no significant pattern emerges. There was no correlation between length of survival and damage scored.

DISCUSSION

The results reported here show that in the artificial metastatic conditions of the lung colony assay it is not possible to increase the colonisation of the lung by C3H tumour cells by subjecting recipient lungs to HBO. If ELD cells are used, a marked decrease in lung colony formation is obtained if the cells are present while HBO treatment is given. There is a difference between these types of tumour cells in their pattern of successful colonization in the lung. Both may be demonstrated, by tritiated thymidine and ^{125}IUdR labelling, to be distributed in vessels throughout the whole lung volume for 12–24 hr after i.v. injection, but while C3H tumour cell nodules develop from arterial emboli to invasive growths throughout the whole lung parenchyma, ELD cell deposits only develop subpleurally and grow through the pleura [14]. Any embolus of ELD cells throughout the rest of the lung fails to survive. (ELD cells *in vitro* grow better at 7% O_2 than at 20% O_2 levels [14]. Subpleural growth would be consistent with the growth of cells at a position in the lung as far removed as possible from the lungs' abundant oxygen supply: when the concentration of dissolved oxygen in tissue fluid is raised at 3 ATA HBO ELD cells colonize less effectively.

The mouse lung, like the rat lung, is extremely sensitive to HBO damage [15] and it might be reasonable to postulate that in the period during and immediately after HBO insult damaged capillary walls in the lung might provide a favourable resting place for tumour cell emboli. We have however obtained no evidence at all for the existence of this phenomenon.

When mice bearing spontaneous tumours, on the other hand, are exposed to HBO treatment, the incidence of pulmonary metastatic deposits is significantly increased over their matched controls. By the time these mice

Table 4. Distribution of lung damaged scored in in relation to metastatic deposits

$\frac{\text{Total score per group}}{n}$ Maximum score per box = 2.0.

	Treatment group	*n*	Haemorrhage into alveoli	Thickened alveolar septae	Congestion	Consolidation	Alveolar macrophages	Lymphocyte cuffing	Emphysema	Collapse
1	Control	22	0.5	0.7	0.7	0.5	0.8	0.2	0.9	0.1
2	Control + metastases	8	0.7	0.6	0.4	0.6	1.0	0.1	1.0	0.1
3	Control no metastases	14	0.4	0.7	0.8	0.4	0.6	0.3	0.8	0.1
4	HBO treated	18	0.6	0.7	0.8	0.2	0.4	0.3	0.8	0.1
5	HBO + metastases	12	0.7	0.7	0.9	0.2	0.2	0.3	0.5	0.2
6	HBO no metastases	6	0.3	0.8	0.5	0.3	0.3	0.4	1.3	0

reach their mean post-treatment survival time, pulmonary damage from HBO would have been repaired [7]. Since the young recipient mice in the lung colony assay gave no evidence for increased settling of C3H tumour cells during transient lung damage with HBO treatment, the increased incidence found in spontaneous tumour-bearing mice cannot be due to increased and more favourable settling in HBO damaged lung. Residual lung damage was no greater in the HBO group.

HBO treatment had no effect on the growth rate of the primary spontaneous primary tumour. This agrees with the consensus in the literature that there is no demonstrable effect of HBO treatment on the growth of transplanted tumours, including C3H [16] and C3HBA mammary tumours [17], C57B1 mammary tumours [6], Strong and DBA mammary tumours [17], Cloudman melanoma and L1210 leukaemia [18]. These results all conflict with the observation of Dettmer *et al.* [19] that exposure to HBO increases the incidence and rate of development of radiation-induced mammary tumours in female rats surviving 400 rad whole-body irradiation, which was interpreted as a possible carcinogenic enhancement effect of oxygen. The experimental design here however involved more complex factors than the simpler investigations of the effect of HBO on tumour growth rate.

The question of the effect of HBO on pulmonary metastasis is more open. One report claims fewer pulmonary metastases in HBO treated mice [6], and two describe no difference in incidence or total number of deposits [7, 17, 18]. The present work reports increased incidence of metastasis in mice exposed to HBO when spontaneous tumours had developed, but no increase in artificial metastases. (Dettmer *et al.* [19] described an increased incidence of second tumours arising after surgical removal of the first primary, but these appear to be second primary tumours at different sites and not secondary metastatic deposits in the lungs).

As the increased incidence of metastases demonstrated here is not related to increased residual HBO damage to the lungs it seems likely that the increased incidence of lung metastases is therefore due to an increased rate of dissemination of tumour cells from the primary tumour. Mechanical factors, such as rubbing or handling a tumour, are known to result in an increased degree of metastatic spread [13, 20], and the increased pressure on a tumour inoculated into the foot pad rather than in the flank results in earlier metastasis [21]. It is believed that the increased metastatic incidence in HBO treated tumour mice is due to a mechanical effect on the primary tumour during compression, or more probably during decompression, resulting in an increased release of tumours cells into the bloodstream.

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