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A STUDY ON THE INFLUENCE OF HYPERBARIC OXYGENATION ON THE RESISTANCE OF TUMOR HOST

—ESPECIALLY ON RETICULOENDOTHELIAL FUNCTION AND ALLOGRAFT REACTION—

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ABSTRACT

A study was carried out on the influence of oxygen at high pressure upon the experimental tumor and the host. The results were as follows;

1) From the observation of 50% survival time in the rats in which the Yoshida sarcoma had been transplanted intraperitoneally, a significant life prolonging effect was noted in the combined treatment of Mitomycin C and oxygen at high pressure (MMC+OHP).

2) Pathological study on the long survived cases disclosed that, in the group treated with MMC+OHP combination, the tumor exudates had disappeared and the collapse of the nuclei of the Yoshida sarcoma cells in the pulmonary capillaries was marked.

3) Although suppression of mitotic index was noted in the group treated with OHP alone the tendency of suppression was significant in the mitotic index of the group treated with MMC+OHP combination.

4) The effect of OHP on the reticuloendothelial function of the host was, except when its function was severely impaired, in the direction of stimulation. The optimum condition of OHP was at 3 ATA and for 30-60 minutes at 24 hours intervals.

5) The optimum condition of OHP served for shortening the mean survival time of allograft on the host. The rejective reaction was the most intense in the group treated with MMC+OHP combination.

INTRODUCTION

Modern therapeutic means of malignant tumors have been centered in surgical, radiological, chemical, and immunological fields, independently or in association. While the target of endeavour in each field is to improve the effect of individual therapeutic means, yet early diagnosis and treatment remain to be the most desirable and dependable for the ultimate cure of the tumors.

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In the surgical field, for example, extensive radical operations for the advanced states of cancer and the techniques of infusing or perfusing anticancer agents have been introduced. In the radiological field, the study for enhancement of tumor susceptibility to radiation has been carried out. In the chemical field, various adjuvant means to chemotherapy have been attempted.

While most of the adjuvant therapies have aimed basically to intensify the destructive effect of the main therapeutic means on tumor tissues by changing the environment of the host, the interest has recently been focused not only in the betterment of postoperative course but also in the ultimate improvement of the effect of chemotherapy or radiotherapy by inducing the propitious changes in the host *per se*. Along these lines the immunological means of adjuvant therapies have attracted the interest of investigators.

Hyperbaric oxygenation (abridged as OHP hereafter) has lately become worth notice as one of the useful adjuvant therapies to radiotherapy and chemotherapy for malignant tumors. High pressure environment had been utilized already in the 17th century for the treatment of caisson disease. In 1662, Henshaw[1] reported on the use of a compression air chamber for the patients with pulmonary diseases. In 1895, Haldane[2] reported on the usefulness of OHP for the treatment of carbon monoxide poisoning. The present enthusiasm in OHP was rooted by Boerema *et al.*[3] in 1956 when they introduced into cardiac surgery the novel concept of "life without blood".

The basic utility of OHP in the treatment of malignant tumors has been as an adjuvant to radiotherapy in which OHP enhances the susceptibility of tumor tissues to radiation. Churchill-Davidson[4] reported in 1955 that OHP at 3 ATA (atmosphere absolute) combined with radiotherapy on the cancers produced favourable results.

It was further reported in 1960 by Emery[5] and in 1965 by Foster[6] that they applied OHP in their clinical cases of radiotherapy in which radiation alone had been ineffective and obtained favourable results.

Reports on the use of OHP, alone or in combination with anticancer agents, have been made in recent years by many authors. In 1964, Boerema[7] reported that OHP alone minimized the pain of the patients in the terminal stage of cancer, though in small number, and that it also was effective in accelerating the necrotic process of melanosarcoma in the upper extremity. Kluft *et al.*[8] in 1964 reported that OHP was effective in temporarily suppressing the growth and metastasis of tumors, and presumed that OHP applied during the operation would serve for preventing the growth of liberated cells.

The combination of OHP with anticancer agents, although there is a debatable ground in the findings of Adams[9], has been reported with favourable results, as noticed in the peport of Krementz *et al.*[10] who stated in 1961 that the application of OHP enhanced the effect of nitrogen mustard and other

alkylating agents.

When the effect of OHP on malignancies is considered, not only its direct effect on the tumor tissue *per se*, but the effect on the host also has to be taken into account. The concept in the therapy of malignancies should consist of both attacking the tumor and improving the conditions on the side of the host.

The study on the growth of tumor from the side of the host was carried out by Stern[11] in 1960. After analyzing the factors in the growth of tumors and the functioning of reticuloendothelial system, he suggested that the disturbance in the function of reticuloendothelial system had much to do with the growth of tumors. The host-tumor relationship was introduced by Herbut[12] in 1956, and by Old *et al.*[13] in 1961 in their suggestion that the proper stimulation of the reticuloendothelial system of the host with B.C.G. or Zymosan could enhance the resistance of the host against cancer.

The reticuloendothelial system, the study of which was initiated by Kiyono[14] and Aschoff[15], was further investigated by Akazaki[16,17] and has been recognized as playing a great role in the maintenance of life while dealing with various metabolism through its phagocytic activity. It has been widely admitted that the resistance of the host against the tumor has a close interrelationship with the reticuloendothelial system and the immunological reactions.

In the viewpoint of reticuloendothelial system and immunological reactions, the present author investigated the influence of OHP on the host from these two directions so that the nature of OHP as an adjuvant therapy to the treatment of malignancies would be clarified.

MATERIALS AND METHODS

Experimental Animal: Both male and female Donryu rats weighing about 100 g were used for the experiment. They were reared with solid feed and tap water at room temperature and humidity.

Experimental Tumor: Yoshida sarcoma which was originally transfered from the 2nd Department of Pathology, Gifu University School of Medicine, was transplanted from generation to generation in the Donryu rats.

Apparatus and Method for Hyperbaric Oxygenation: An experimental hyperbaric chamber which has been designed in the 1st Department of Surgery, Nagoya University School of Medicine, was used throughout the experiments. As shown in Fig. 1, the main constituent of the hyperbaric chamber is a cylinder with the diameter of 560 mm and the length of 1,115 mm. Positive pressure was always applied with pure oxygen. The experimental animals were placed in the chamber and about one minute was required to replace the

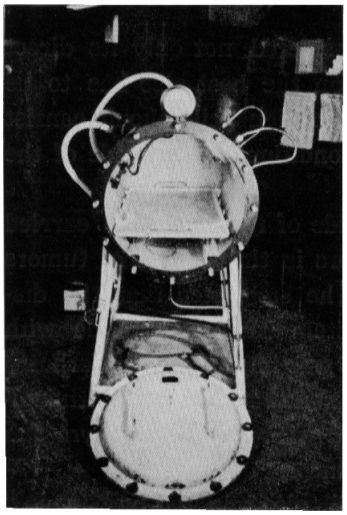

FIG. 1. The hyperbaric oxygen chamber in this study.

air in the chamber with pure oxygen before the pressurization with pure oxygen could be started. The pressurization was practised at the rate of 0.2 kg/cm^2/min and ten minutes was necessary before the internal pressure reached 3 ATA. Release of the pressure was practiced as well at the rate of 0.2 kg/cm^2/min.

Administration of Anticancer Agent: The anticancer agent used during the experiment was Mitomycin C (MMC) and this was injected intraperitoneally in the single dose of 0.5 mg/kg.

Observation of Survival Period: The treatments were commenced on the 3rd day after the intraperitoneal transplantation of the 5×10^6 Yoshida sarcoma cells. Four groups, ten each, were served for the control, the group treated with single OHP of 30 minutes, the group treated with single MMC administration, and the group treated with MMC and OHP combination. MMC was given only on the 3rd day of transplantation while OHP treatment was repeated from the 3rd day to the day of death at the rate of once a day and with 30 minutes duration each. The individual which lived longer than 19 days after transplantation of the sarcoma cells was denoted as the case of long survival.

Histopathological Observation: The cases of long survival were slaughtered on the 19th day after transplantation and served for the observation of abdominal organs and for histopathological investigation of the lung tissues.

Measurement of Mitotic Index: Initially the intraperitoneal transplantation of the 5×10^6 Yoshida sarcoma cells, and from the 3rd day, ascites was aspirated at regular intervals by using a fine glass pipette to provide three of Giemsa stained preparations on each specimen. On each preparation, the number of mitotic cells per 2,000 of Yoshida sarcoma cells was counted to calculate the mitotic index. Twenty five rats were divided into 5 groups, namely, the control group, the group treated with single OHP of 30 minutes duration, the group treated three times with OHP of 30 minutes duration at 12 hours intervals, the group treated with single MMC administration, and the group treated with MMC and three times of OHP of 30 minutes duration. The treaments with both OHP and MMC were commenced on the 3rd day after the intraperitoneal transplantation.

Assessment of Reticuloendothelial Function: Carbon clearance method as has been reported by Biozzi *et al.*[18] was used for the assessment of reticuloendothelial function. This method consists of the following procedures. Powdered gelatine is dissolved into physiological saline to make 1% solution, which is then adjusted to pH 7.2 by addition of ammonia. The solution is kept at 37°C. One ml of the commercial Pelikan Black Ink is mixed with 2.3 ml of the stored gelatine solution immediately before its administration to the animal. The material thus prepared contains 30 mg of carbon particles in each 1 ml. The carbon suspension is injected into the femoral vein of the rats in the amount of 0.52 ml (16 mg of carbon) per 100 g of body weight. 0.02 ml of blood is sampled accurately using a glass pipette from the retro-orbital venous plexus at 3 and 15 minutes after the injection. The blood sample is then mixed with 4 ml of 0.1% sodium carbonate solution. Using the blood sample obtained before the injection as the control, the amount of carbon in the sample is measured with a photospectrometer at the wave length of 610 mμ.

The function of reticuloendothelial system of the rat is assessed from the degree of carbon clearance as phagocytic index. The following formula is given for the calculation of phagocytic index "K";

$$K = \frac{\log C_3 - \log C_{15}}{12}, \text{ where}$$

C_3: The amount of carbon in the circulating blood at 3 minutes after the injection.

C_{15}: The amount of carbon in the circulating blood at 15 minutes after the injection.

The depression[19] of reticuloendothelial function was also artificially produced by using methyl palmitate, which was dissolved in Tween 20 and was made a suspension by adding 5% dextrose and homogenizing for longer than 10 minutes in a microhomogenizer. This suspension of methyl palmitate was administered to a group of rats once a day for two consecutive days in the dose of 70 mg/100 g of body weight.

Immunological Experiment with Allograft: Wistar strain rats were used as the donor, and Gifu strain Donryu rats as the recipient. The body weights in both strains ranged between 150 and 200 g.

Allograft was performed according to the method described by Medawar *et al.*[20][21]. The donor Wistar strain rats were first anesthetized with 10% urethane and the hair at the center of the back was widely removed. Two days later, the skins of rats were removed with the suprapanicular technique in the rectangular surface of 4×6 cm. This technique of skin removal was aimed not to injure the capillaries in the paniculus cavernosus. A square skin

graft of 1.8×1.8 cm was made out of the removed skin and was kept in physiological saline. The recipient Donryu rats were treated in the same way in removing the layer of skin of 2×2 cm square surface on the median line of the back. After confirming that there was no bleeding at the floor of skin removal in the recipient rat, the graft was sutured to the recipient at the border of skin so that the surface of the graft maintained an appropriate tension. After the graft was sutured, the wound was covered by a piece of aseptic gauze in order to maintain tranquility, pressure, and adequate humidity at the site of grafting. The condition of the graft was inspected once a day at regular intervals.

The judgement for the rejection of the graft was based on the time when the rejective reactions such as necrosis or bleeding were observed in more than 50% of the surface of the graft. The grafts which showed necrotic signs before the 5th day of the skin transplantation were excluded from the present experiment.

RESULTS

Survival Period: All the rats, into whose peritoneal cavities were introduced 5×10^6 cells of the Yoshida sarcoma and who were left without further treatment as the control group, died on between the 5th and the 7th day. The 50% survival period of this group was 6.2 days.

All the rats in the group of OHP treatment alone died on between the 5th and the 15th day after the transplantation, with one exception of rat which lived longer than 19 days. The 50% survival period of this group was 7.5 days.

In the group of MMC treatment alone, the rats died on between the 7th

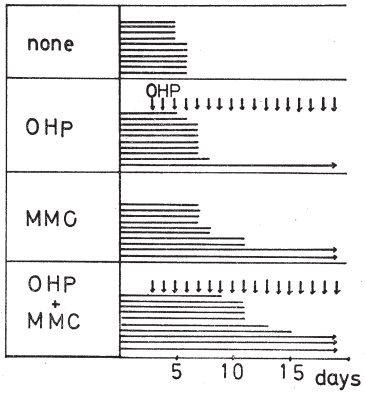

FIG. 2. Survival perid of the four groups treated with different way.

TABLE 1. 50% Survival Period of the Four Tumor-bearing Groups of Rats

Treatment	Survival period (Days)
None	6.2
OHP	7.5
MMC	9.5
MMC+OHP	13.0

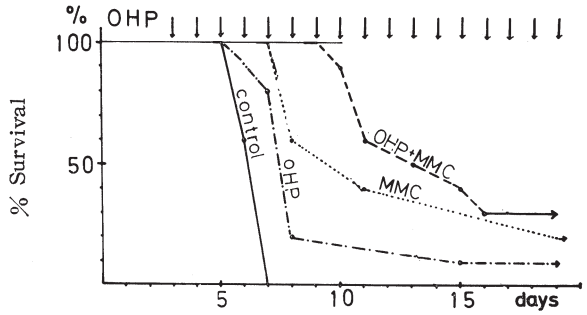

FIG. 3. Survival rate of the four tumor-bearing groups of rats.

and the 11th day except 2 exceptionally long survival cases. The 50% survival period of this group was 9.5 days.

In the group of MMC + OHP combined treatment, there were three cases which lived prolongedly, but otherwise they died on between the 9th and the 15th day, giving the 50% survival period of 13.0 days (Figs. 2, 3 and Table 1).

Histopathological Observation: The animal which lived longer than 19 days in the group of OHP treatment alone showed no cancerous appearance in the abdomen to the naked eye. Histological examinations of the lungs revealed a marked picture of emphysema, in which significant enlargement of alveoli and thin or torn alveolar walls were observed. There were some pulmonary capillaries which were full of Yoshida sarcoma cells (Figs. 4 and 5).

In the long survival cases of the group treated with MMC alone, except the pulmonary findings of packed Yoshida sarcoma cells in the capillaries, there were no cancerous changes in the peritoneal cavity.

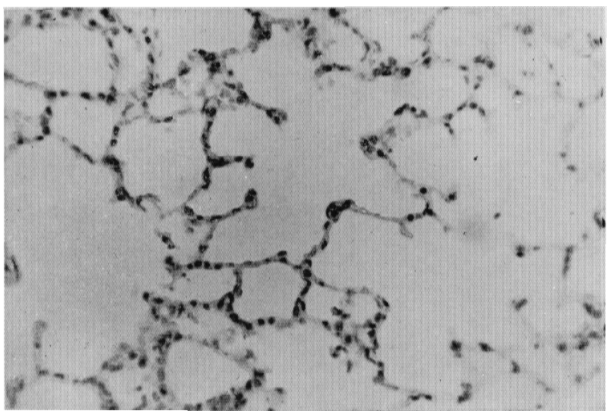

FIG. 4. The finding of the alveoli of a tumor-bearing rat treated with OHP: Alveolar walls are distended with the presence of pulmonary emphysem. ×400(HE)

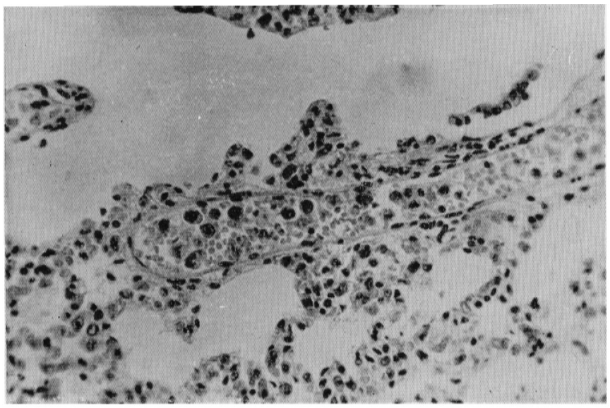

FIG. 5. Pulmonary capillaries of a tumor-bearing rat treated with OHP: The lumen is fulled with Yoshida sarcoma cells. ×600(HE)

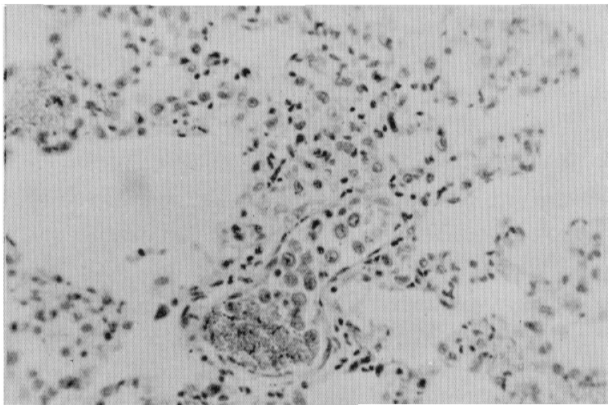

FIG. 6. Yoshida sarcoma-bearing rat trated with MMC+ OHP combination: The Pulmonary capillaries are full of the Yoshida sarcoma cells, of which nuclei and protoplasma are specically collapsed and degenerated in great numbers. ×600 (HE)

In the group of MMC + OHP combined treatment, there were no changes in the abdominal cavity. The histological examination of the lungs revealed the picture of emphysema, and besides, the pulmonary capillaries were full of Yoshida sarcoma cells, of which nuclei and protoplasma were specifically collapsed and degenerated in great number (Fig. 6).

Changes in Mitotic Index: While in the control group the mitotic index consistently increased, in all the groups treated differently, the index showed a significant tendency of decrease. The most significant of all was the group

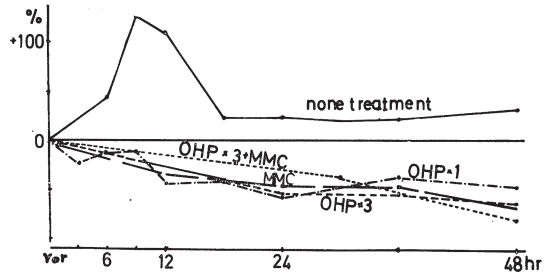

FIG. 7. Changes in mitotic index.

of MMC + OHP combined treatment (Fig. 7) in which the mitotic index at the 48th hour after the treatment showed a marked decrease compared with other treated groups.

Change of Reticuloendothelial Function in Healthy Rats: The skin and the eye balls became black-tinted after the injection of the carbon suspension in the dose of 16 mg/100 g of body weight, but the tint was cleared to normal color at 30 minutes after the injection. The hepatic tissue at 30 minutes after the injection showed that many carbon particles were taken up by the stellated cells of Kupffer (Figs. 8, 9 and 10).

The amount of carbon particles in the blood after the injection of the suspension decreased rapidly until the 3rd minute and slowly afterward. The carbon clearance measurement was based on the amount of carbon in the blood specimen obtained 3 and 15 minutes after the injection.

The phagocytic index "*K*" of the healthy 19 rats ranged between the highest value of 0.056 and the lowest one of 0.037, as shown in Fig. 11, and the mean value was 0.0471.

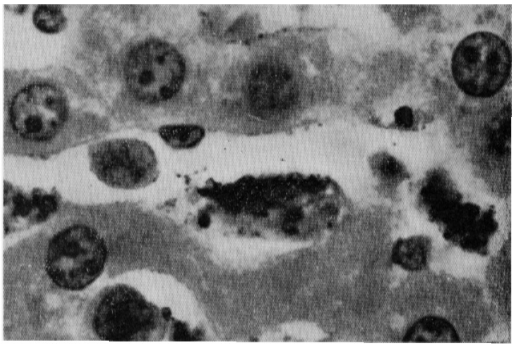

FIG. 8. Hepatic tissue at 30 minutes after the injection of carbon particles in a dose of 16 mg/100 g of body weight: Kupffer cells are seen in phagocytic state. ×1,000(HF)

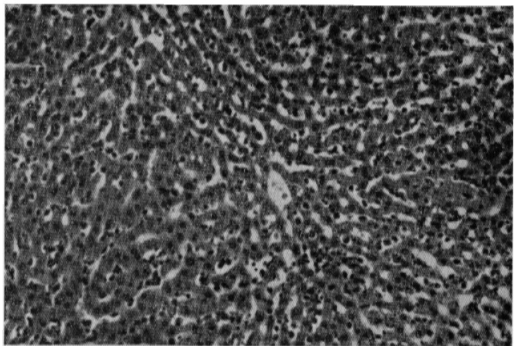

FIG. 9. Hepatic tissue at 30 minutes after injection of carbon particles in a dose of 16 mg/100 g of body weight. The black dots represent the Kupffer cells with carbon particles. ×400(HE)

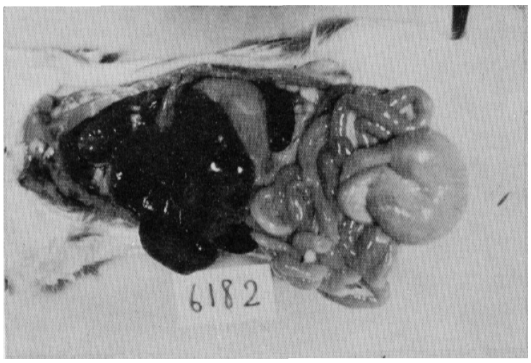

FIG. 10. Intra-abdominal organs at 30 minutes after the injection of carbon particles in a dose of 16 mg/100 g of body weight: The liver and spleen which have taken up the carbon particles appear black.

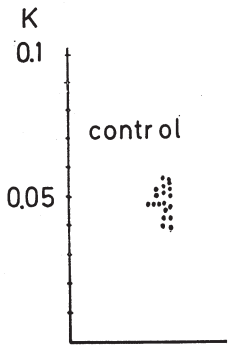

FIG. 11. Phagocytic index (K).

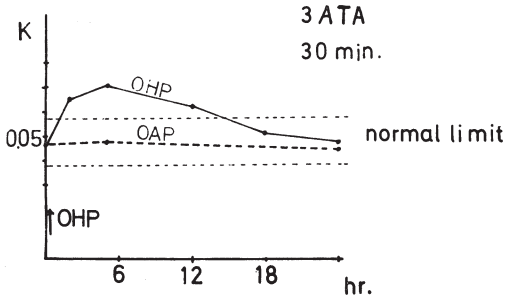

FIG. 12. Changes in phagocytic index.

The result of measurement of K values at regular intervals in the healthy rats which were treated with oxygen at atmospheric pressure (OAP) of 30 minutes duration showed that the values fell within the normal range (Fig. 12).

When the K values were measured at regular intervals in healthy rats after they were treated with OHP at 3 ATA for 30 minutes, they consistently increased until the 5th hour reaching the maximum value of 0.07, then they gradually decreased so that they fell within the normal range between the 18th and the 24th hour.

After the healthy rats treated with OHP at 3 ATA of 30 minutes duration, which was repeated 3 times at 3 different intervals of 12, 24 and 48 hours, the K value were obtained at 5th and 24th hour after the last OHP treatment. The highest figure of 0.100 was obtained at the 5th hour in the group treated at 24 hours intervals. The lowest figure of 0.069 was obtained at the 5 hour in the group treated at 48 hours interval. K values obtained at the 24th hour

after the last treatments took more or less similar figures among the three groups of OHP treatment at different intervals (Table 2).

TABLE 2. The Value of Phagocytic Index After the Three Times Treated with OHP at 3 ATA of 30 Minutes Duration

Interval (hr)	K	
	After 5 hr	After 24 hr
12	0.079	0.054
24	0.100	0.050
48	0.069	0.060

Figs. 13, 14 and 15 show the K values in the three groups of rats to whom OHP at 3 ATA was applied for 5 times but with different durations as 30 minutes, 1 hour, and 2 hours. The interval of application was the same in the 3 groups at 24 hours. The K value was obtained in the 5th hour after each application.

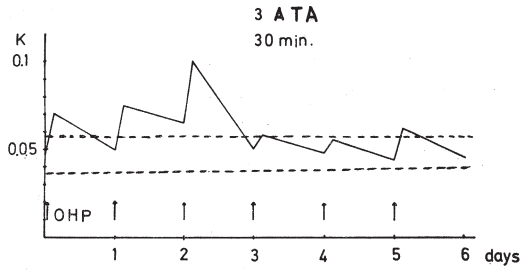

FIG. 13. Changes in phagocytic index.

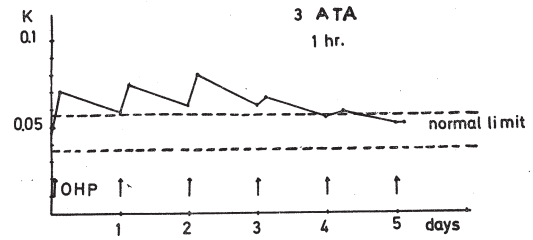

FIG. 14. Changes in phagocytic index.

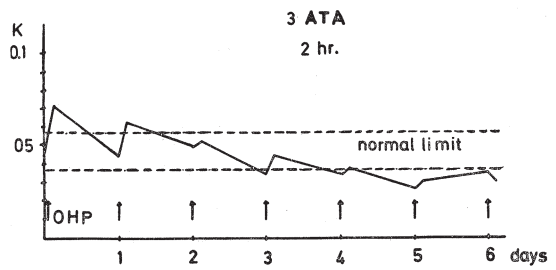

FIG. 15. Changes in phagocytic index.

In the group of 30 minutes OHP treatment, the K value was elevated until the 3rd application and reached the maximum of 0.10, then it fluctuated in the normal range (Fig. 13).

In the group of 1 hour OHP treatment, the K values were elevated until the 4th application, though less markedly than in the group of 30 minutes treatment, and reached the maximum value of 0.08 after the 2nd application of OHP (Fig. 14).

In the group of 2 hours OHP treatment, the K values were slightly elevated, but not exceeding the normal range very far, until the 2nd application, then gradually decreasing to below the normal (Fig. 15).

In the group of 30 minutes OHP treatment, which was repeated 5 times at 24 hours intervals, some of the rats were given OHP at 2 ATA and others were given OHP at 3 ATA. The K values obtained 12 hours after each application are shown in Figs. 16 and 17. The tendency of K values in those treated at 3 ATA was almost the same as those shown in Fig. 13, namely, being elevated until after the 3rd application and staying in the normal range thereafter (Fig. 16). The K values in the 2 ATA group were slightly elevated until after the 5th application but in less noticeable degree than in the 3 ATA group. There was not found any tendency of K values to decrease below the normal range as in the group treated with OHP at 3 ATA of 2 hours duration (Fig. 17).

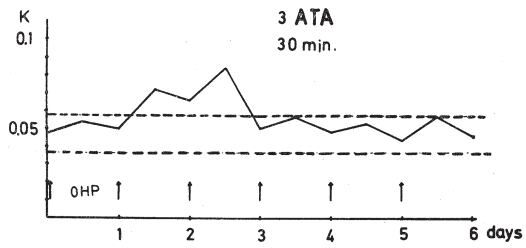

FIG. 16. Changes in phagocytic index.

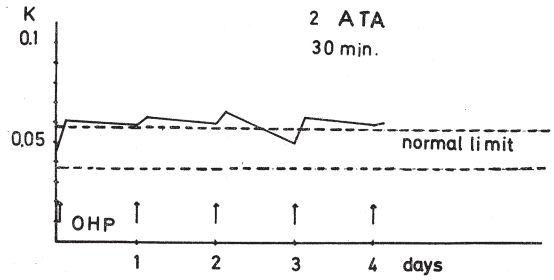

FIG. 17. Changes in phagocytic index.

Table 3 shows the K values in the group of rats whose reticuloendothelial function was once depressed artificially by methyl palmitate, and on which 30 minutes of OHP at 3 ATA was applied 3 times at 24 hours intervals. It was revealed that OHP treatment did not alter the course of reticuloendothelial depression significantly.

TABLE 3. Phagocytic Index in the Control Group Treated with OHP Alone, the Group Treated with Methyl Palmitate and the Group Treated with Methyl Palmitate and OHP

Treatment	K
OHP×3	0.100
Methyl palmitate	0.032
Methyl palmitate+OHP×3	0.035

Change of Reticuloendothelial Function in Tumor-bearing Rats: The K values obtained every day in the rats after the transplantation of the Yoshida sarcoma cells are shown in Fig. 18. The K values continued to decrease after the transplantation and reached the lowest value just before the death.

The commencing period of the application of OHP at 3 ATA of 30 minutes duration on the tumor-bearing rats was divided into three categories: immediate post-transplantation period, the 3rd day, and the 5th day. The K values which were obtained immediately before and 5 hours after the OHP treatment are shown in Figs. 19, 20 and 21. In the group in which OHP was commenced immediately after the transplantation, the K value continued to be elevated until the 3rd day, and fluctuated in the normal range thereafter (Fig. 19).

In the group in which OHP was commenced on the 3rd day of transplanta-

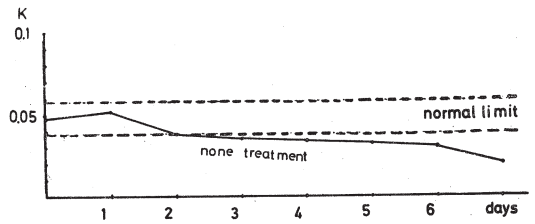

FIG. 18. Changes in phagocytic index of the Yoshida sarcoma-bearing rats.

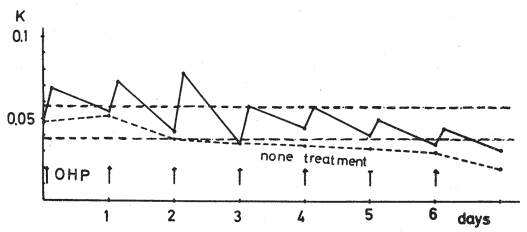

FIG. 19. Changes in phagocytic index of the Yoshida sarcoma-bearing rats.

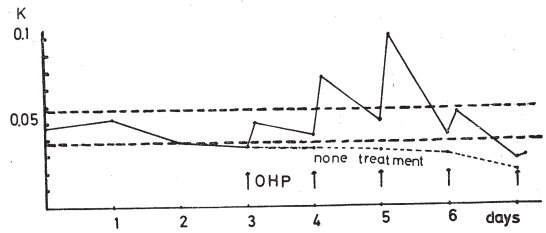

FIG. 20. Changes in phagocytic index of the Yoshida sarcoma-bearing rats.

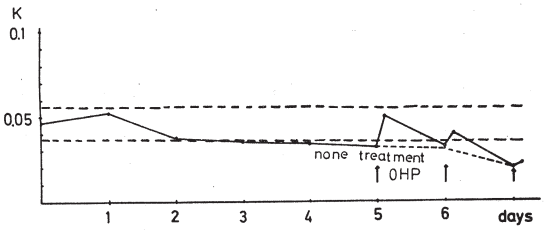

FIG. 21. Changes in phagocytic index of the Yoshida sarcoma-bearing rats.

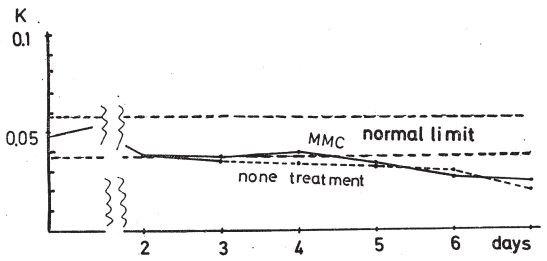

FIG. 22. Changes in phagocytic index of the Yoshida sarcoma-bearing rats.

tion, the K value elevated in the 2nd and the 3rd measurement, the latter taking the highest value of 0.100, then it decreased rapidly (Fig. 20). In the group in which OHP was commenced on the 5th day, the K value was not elevated at all, and it followed the similar course to the control group (Fig. 21).

In the rats treated with MMC in the 60th hour after the transplantation of the Yoshida sarcoma, the K value was measured at regular intervals. As shown in Fig. 22 the K value was not elevated at all, virtually with no difference from the control.

In the group of MMC + OHP combined treatment, in which MMC was administered just before the treatment of OHP at 3 ATA of 30 minutes duration was commenced on the 3rd day of transplantation, the K values took the

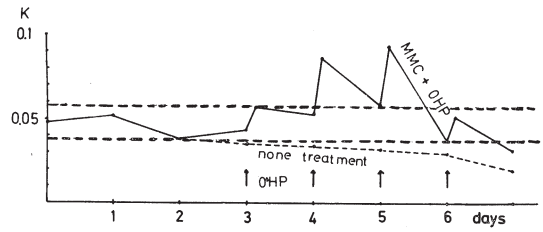

FIG. 23. Changes in phagocytic index of the Yoshida sarcoma-bearing rats.

similar course to that in the group in which OHP alone was applied from the 3rd day of transplantation (Fig. 20) as shown in Fig. 23. The K value gradually increased from the 1st toward the 3rd measurement, the latter taking the highest figure of 0.10.

Immunological Experiment with Allograft: Three groups of non-tumor-bearing rats were served for the study of survival time of allograft; namely, the control group, the group treated 3 times with OHP at 3 ATA of 30 minutes duration before the grafting, and the group treated 3 times with OHP at 3 ATA of 1 hour duration. The survival time of the graft in these 3 groups of rats is seen in Fig. 24. The mean survival time of these groups is summarized in Table 4. The control group had the mean survival time of 11.2 ± 0.8 days, the 30 minutes OHP group 9.0 ± 0.5 days, and the 1 hour OHP group 9.3 ± 0.9 days. Between the two OHP groups there was no significant difference, but there was a clear difference between the control and OHP groups.

Allograft experiment was then carried out on the Yoshida sarcoma-bearing rats, in the number of 5×10^4 cells. They were divided into 4 groups; the control group,

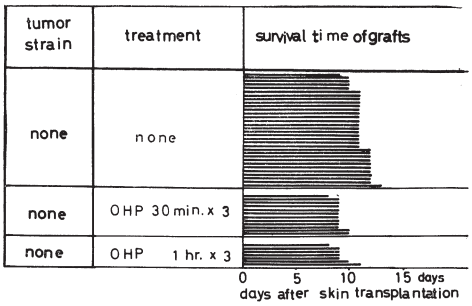

FIG. 24. The effect of OHP on mean survival time of allograft.

TABLE 4. The Effect of OHP on Mean Survival Time of Allograft

Tumor strain	Treatment	No. of rats	Mean survival time of grafts (days)	
none	none	32	11.2±0.8	$> P < 0.05$
none	OHP 30 min×3	12	9.0±0.5	
none	OHP 1 hr×3	7	9.3±0.9	

TABLE 5. The Mean Survival Time of the Grafts on Yoshida Sarcoma Bearing Rats

Tumor strain	Treatment	No. of rats	Mean survival time of grafts (days)
Yoshida sarcoma	none	8	13.3±0.7
Yoshida sarcoma	OHP	10	12.5±0.5
Yoshida sarcoma	MMC	7	13.2±0.7
Yoshida sarcoma	OHP+MMC	10	11.5±0.9

the group treated 3 times with OHP of 30 minutes alone, the group treated with MMC alone, and the group treated with MMC + OHP combination. The treatment with MMC or OHP was performed before the grafting. Table 5 summarizes the mean survival times of the graft in the 4 groups.

While the mean survival time in the control group was 13.3 ± 0.7 days, all

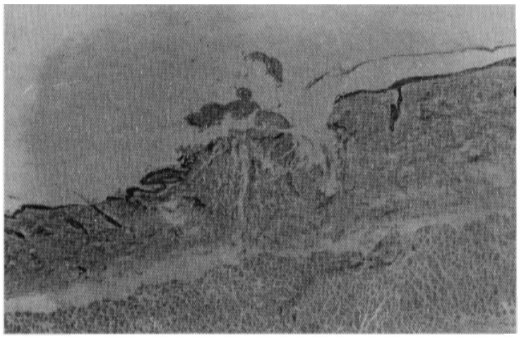

FIG. 25. Non-treated case: Immediately after the skin transplantation. The panicula cavernosus is preserved perfectly well at the border of the host and the graft. A part of the suture is seen. ×100(HE)

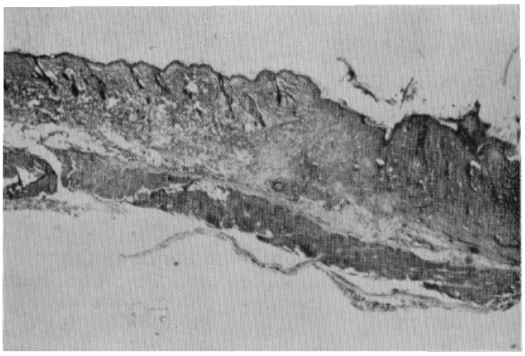

FIG. 26. Non-treated case: Nine days after the skin transplantation. The brims of the host and the graft are adherent. ×40(HE)

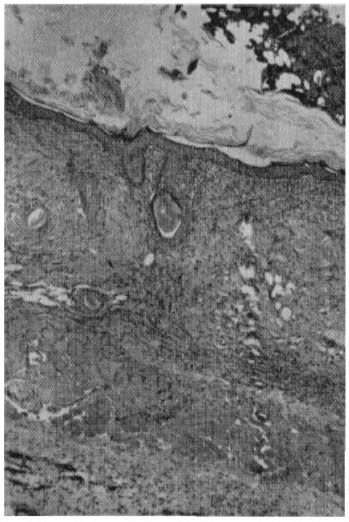

FIG. 27. Non-treated case: Nine days after the skin transplantation. Cellular infiltration is seen around the graft mainly consisting of lymphoid cells. ×100(HE)

other treated groups had more or less shortened survival times with minor variations. While the mean survival time in the MMC group did not so much differ from that in the control group, the group treated with MMC + OHP combination had a fairly shortened mean survival time, 11.5 ± 0.9 days, in comparison with the control.

Histological observations of these grafts revealed that the paniculus cavernosus immediately after the grafting was preserved perfectly, as shown in Fig. 25, suggesting the success of grafting. Macroscopic observation of the graft on the 9th day of grafting revealed an adhesion of the brims of the graft and the skin of the host (Fig. 26), whereas the microscopic pictures were the infiltration of lymphoid cells, mast cells, and plasma cells, around the graft (Fig. 27). The pictures of the graft on the 15th day after grafting were those of rejection represented by overall necrosis (Fig. 28). Figs. 29 and 30 show the grafts of a non-treated case and an OHP treated case. While the non-treated control is seen to be well inserted, the OHP treated case shows the evident picture of rejection.

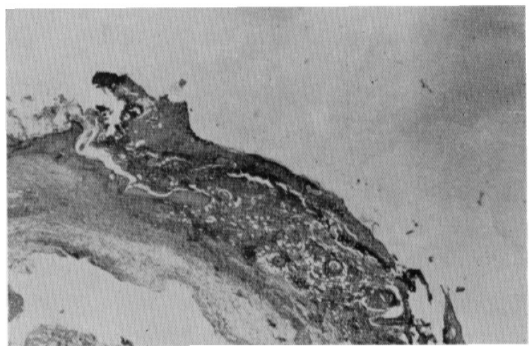

FIG. 28. Non-treated case: Fifteen days after the skin transplantation. The graft shows hyaloid appearance, clearly mainfesting the rejection, being surrounded with marked cellular infiltration. ×40(HE)

FIG. 29. Non-treated case: Eleven days after skin transplantation.

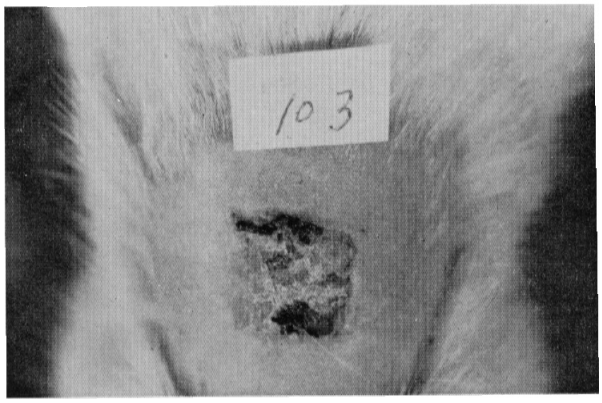

FIG. 30. The finding of the graft of a case treated with OHP: Eleven days after skin transplantattion.

DISCUSSION AND CONCLUSION

Although there has not been established a definite theory on the utility of OHP as an adjuvant therapy to cancer chemotherapy, its enhancement of the tumor susceptibility to radiation is well recognized and has been applied clinically. In the recent years, on the other hand, there have appeared many reports relating the effectiveness of OHP alone or its combined use with cancer chemotherapy.

Pace *et al.*[22] reported on the destruction of HeLa cells under various conditions of oxygen concentration in their experiment in tissue culture. Rückert and Müller[23] as well reported on the suppression of the proliferation of HeLa cells in the various oxygen environments. Asano *et al.*[24] of this Department, in their experiment in tissue culture of FL, HeLa, and Yoshida sarcoma cells, reported on the effectiveness of OHP for the suppression of their proliferation

particularly of Yoshida sarcoma cells. Mori[25] of this Department, in his results of the experimental therapy of tumor-bearing animals with OHP treatment alone, reported that the life prolonging effect could not be expected of OHP treatment alone. In the present experiment also, the OHP treatment alone resulted in minor degree of life prolonging evidence compared with the control, with only one exceptional case of long survival. The life prolonging effect of OHP alone revealed to be much inferior to MMC treatment alone. The most significant treatment for life prolongation in the present experiment was the MMC + OHP combination, which rendered the 50% survival time of 13.0 days, significant enough compared with the control, the MMC group, and the OHP group. Further support is endowed by the histological findings among the long survival cases, in which the collapse of the nuclei of the Yoshida sarcoma cells in the pulmonary capillaries was more remarkable in the group of MMC + OHP combined treatment than in the group of OHP treatment alone. The marked emphysematous picture in the lungs of long surviving cases is considered to be due to the repeated applications of OHP. Takeichi[26] of this Department related that he observed bleeding into alveoli, thickening of alveolar walls, and localized emphysema, in his experimental animals treated 5 times with OHP at 3 ATA of 2 hours duration.

The incidence of 50% survival time and the collapse of nuclei of tumor cells corresponds well further with the result of the measurement of mitotic index, which was depressed the most in the group of treatment with MMC + OHP combination among all the treated groups.

All the above result still fails to denote whether the cancerocidal effect of MMC was enhanced by OHP or the resistance of the tumor host *per se* was influenced favourably by OHP. This question necessitated further study of the effect of OHP on the side of host, measuring the reticuloendothelial function as one of the parameters.

There has been long postulated the possibility that some form of reaction of the reticuloendothelial system is present against the assault of malignant tumors. Halpern[27] reported on his experiment using Ehrlich's tumor that the reticuloendothelial system reacted differently to the kind of tumor and the site of transplantation. In 1938, Druckrey[28] made a contradictory report on the anticancer activity of reticuloendothelial system. The depressant effect of cortisone on the phagocytic activity of reticuloendothelial system is generally admitted since it was reported by Nicol and Bilbey[29], but it is also reported that steroid hormones administered to the patient with breat cancer exert anticancer effect by assisting the increase of collagen tissue around the tumor. Stern[11] and Old *et al.*[13] hold that the reticuloendothelial function has a great influence on the resistance of the host against tumors, and that the stimulation of the function generally suppresses the growth of tumors and enhances the

anticancer activities in the host. On the other hand, Berg[30] who studied the reticuloendothelial system histologically proposed that the increase in the number of histiocytes did not mean anticancer response. However, there is recently a strong tendency to believe that the increase in interstitial cells in the regional lymphnode in breast cancer is the anticancer manifestation of the host[31)32)]. Takeichi[26] of this Department examined the histology of metastatic lymphnodes and noted the increase in sinus histiocytes and plasmacytes belonging to the reticuloendothelial system after OHP application. In view of the aforementioned background, the present author assumed the current concept of anticancer response of living body in the level of reticuloendothelial system in the following part of the discussion.

Assessment of the function of reticuloendothelial system is based on its phagocytic efficiency of foreign bodies, and there are several means for its detection[33]. The direct means of detection is to observe the phagocytic state of the system histologically after injecting foreign bodies, and the indirect means is to measure the degree of clearance of the injected foreign bodies from the circulating blood, the latter being represented by carbon clearance method and congo red method[34].

While there is a report by Ninomiya[35)36)] that with the congo red method of Adler-Reimann the phagocytic efficiency is influenced by the state of hepatic function as the pigment is due to be excreted into the bile juice, the carbon clearance method reported by Halpern *et al.*[37] in 1953 has been recommended to be ideal, since the carbon particles of commercial Pelikan Black Ink is homogeneous and free from any toxicity with the dose of 16 mg/100 g of body weight. The carbon clearance method used in the present study has enabled the course of experiment to run smoothly throughout, without forming clusters in the blood or forming emboli in the capillaries.

The fact that the treatment with OHP at 3 ATA for 30 minutes stimulates the function of reticuloendothelial system was proven in the comparison of K values with those in OAP treatment (Fig. 12), although the optimum condition of OHP for the stimulation of reticuloendothelial function was left still unclear. The optimum condition of OHP for reticuloendothelial function was to be studied in relation to the interval, the duration, and the pressure of each application.

As regards the interval of repeated OHP treatment, 3 different intervals were examined by measuring the K value (Table 2), and the highest K value was found in the treatment at 24 hours intervals. This suggested the optimum interval of OHP application to be once a day.

The duration of each application was perused in the results of the K values, which are summarized in Figs. 13, 14, and 15. It was suggested that either 30 minutes or 1 hour as the duration of OHP application was able to elevate the K value until the 3rd or 4th application. This was particularly evident

after the 3rd application of 30 minutes duration in which case the K value took the highest figure of 0.10. In the 2 hours OHP group, the K value was not elevated except after the 1st application. In all the 3 groups, after the K took the maximum values, the more often the OHP treatment was repeated the lower went the resultant K values. All the above results suggest that the OHP treatment repeated up to 3 or 4 times with the duration of 30 minutes to 1 hour each would be effective in stimulating the reticuloendothelial function, but when the more often repeated there is no more stimulation of the function. This is considered to be an interesting finding in association with the problem of oxygen tolerance of the living body.

The optimum pressure of OHP could be discussed on the K values obtained in the groups treated with 2 ATA and 3 ATA of OHP independently, both with the uniform duration of 30 minutes and at 24 hours intervals. It is seen in Figs. 16 and 17 that the K value in the treatment at 3 ATA took higher figures than in the treatment at 2 ATA.

The overall suggestion of the above discussion leads to the assumption that the optimum condition of OHP to stimulate the functioning of reticuloendothelial system is in its application at 3 ATA and of 30 to 60 minutes duration, and up to a few times at 24 hours intervals.

Sasamoto[38] pointed out in 1966 in his experiment in oxygen toxicity using rats, that OHP treatment at 3 ATA for longer than 2 hours resulted in symptoms of oxygen toxicity in more than 50% of the cases, but not to the degree of mortality. Boerema[39] suggested in 1961 that the limit of the duration of OHP at 3 ATA in the treatment of human beings was up to 2 hours.

The most effective commencing time of OHP treatment on the tumor-bearing rats was assessed from the view point of K values. It was suggested that the commencement of OHP treatment befor the 3rd day of sarcoma transplantation contributed to the elevation of K values, but the commencement after the 5th day would no longer enhance the function of reticuloendothelial system (Fig. 19, 20, 21).

The changes in the K values in the Yoshida sarcoma-bearing rats (Fig. 18) are almost similar to the results obtained by Tsuji[40] who measured the function of reticuloendothelial system in 1965 using the congo red method. It was also suggested that OHP treatment would fail to restore the reticuloendothelial function any longer in its extremely depressed stage, as represented by the lowest K value of 0.02 immediately before the death of the tumor-bearing rats. This was substantiated by the failure of OHP treatment to restore the reticuloendothelial function which had been impaired artificially by methyl palmitate (Table 3).

In regard to the relation between the reticuloendothelial function of the host and the quantity of transplanted tumor cells, there is the report by Old

et al.[13] in 1961 that when Sarcoma 180 was transplanted to Ha/ICR Swice mouse the reticuloendothelial function as measured by carbon clearance appeared to be stimulated by small quantity of the transplanted sarcoma cells while depressed by large quantity of cells.

The comparison of MMC treatment alone and MMC + OHP combined treatment in relation to the reticuloendothelial function, as suggested by the result that the combined treatment elevated the K values to the similar extent to those by OHP treatment alone while MMC treatment alone consistently depressed the K values, contributed to the assumption that OHP effected favourably on the host in MMC therapy.

The theoretical and technical aspect of allograft was described in 1944 by Medawar[21]. The advantage of the technique is in its relative simplicity in the procedure, abundance in material, readiness for assessment of the insertion, and the tolerance of the animals to the surgical assault. Lately it was pointed out that the allograft reaction was the most sensitive manifestation of the immunological response of the host, that is in other words the delayed type of antigen-antibody reaction. The experiment with allograft in the present study was aimed not only to substantiate the findings in the host resistance detected by carbon clearance test but also to serve for the investigation of immunological state of the tumor host.

The mean survival time of the control allografts in this experiment corresponds fairly well with the results of other reporters summarized in Table 6. The direct influence of OHP on the site of allograft was avoided in this experiment by applying OHP before the grafting procedure was practiced, since it has been pointed out by McFarlane *et al.*[41] in 1966 that OHP influences the transplanted skin directly and prevents the development of necrosis of the graft.

The grafting in the present study was practiced first on the normal rats of which reticuloendothelial function had been enhanced by OHP of optimum conditions. There was a significant difference between the control and the pre-treated groups in the mean survival time of allograft (Table 4), though there was no difference in the survival time between 30 minutes and 1 hour of

TABLE 6. Mean Survival Time of Graft According to Different Reporters

	Mean survival time of grafts (days)	Animal
Billingham, R. E.[20]	11.0±0.3	mouse
G. Fujii[50]	11.0	mouse
U. Ishibashi[49]	9.4	mouse
T. Akiyama[48]	10.2±0.3	mouse
K. Hanai[47]	11.0±0.3	rat
S. Fujinami[44]	11.0	rabbit
T. Okada	11.2±0.8	rat

the duration of pretreatment. These findings may be considered in parallel with the findings of Fujinami[44] in 1967 that treatment of the liver with X-ray radiation before skin grafting intensified the rejective reaction of allograft. The pretreatment with OHP may as well stimulate the antibody-producing efficiency of the host.

The result of allograft application to the tumor-bearing rats, which is summarized in Table 5 as for the mean survival time of allograft, reveals that the mean survival time of the control was fairly prolonged over that of normal rats. This finding is supported by the general statement that the productivity of antibodies is lowered in the tumor host[42)43)].

The rejective reaction in the groups treated with MMC alone and OHP alone was not intensified more than in the control group, but it was enhanced to certain degree in the group treated with MMC + OHP combination.

It is considered therefore that the OHP treatment has not only enhanced the anticancer effect of MMC on the tumor cells per se but also reinforced the antibody productivity of the tumor host.

The relationship between the state of allograft and the resistance of host was dealt with in the reports by Fujinami[44] in 1967 and by Ozawa[45] in 1968 in their experiments using rabbits. They suggest that the enhancement of the host resistance could suppress the growth of tumor, resulting in the rejection of allografts, while on the other hand the reduction of the host resistance delays the rejective reaction. Ishibashi[46] reported in 1954 that repeated injection of bone marrow cells of the normal C_3H strain mouse to the mouse of the same strain with breast cancer resulted in apparent prolongation of life, and further that the allograft from the C 57 BL mouse on this treated mouse showed intense rejective reaction as in the normal mouse.

It is clear from all these reports and the results of the present experiments that the OHP treatment in the optimum condition exerts a stimulative effect on the reticuloendothelial function and the intensification of rejective reaction of allograft in the immunological plane. The nature of the mechanisms involved in these effects is not yet clear and there remains a room for debate whether the experimental results are the direct manifestation of the reinforced resistance of the host. In any event, it can be assumed that the OHP treatment had a favourable influence on a certain factor of host resistance.

In conclusion of the present experimental study, the OHP treatment combined with MMC administration can intensify the anticancer effect, and the optimum condition is to be sufficed by repeated application of OHP at 3 ATA of 30 minutes to 60 minutes duration and at 24 hours intervals. Under this OHP condition, not only the apparent effect on the tumor cell *per se* but also an advantageous influence on the host could be expected.

SUMMARY

It has been experimentally proved that oxygen at high pressure applied under optimum conditions not only effects on tumor cells *per se* but also influences on the host favourably, through stimulating reticuloendothelial function and enhancing resistance of the host.

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REFERENCES

1) Onji, U., Yoshiya, I., Yoshikawa, K., Ota, M., and Awano, M., Hyperbaric oxygen therapy, Nagai publisher, 1967, 1 p. (in Japanese)
2) Haldane, J., The relation of the action of carbonic oxide to oxygen tension, *J. Physiol.* (London), **18**, 201, 1895.
3) Boerema, I., Mayne, N. G., Brummelkamp, W. K., Bouma, S., Mensch, M. H., Kamermans, F., Hauf, M. S. and Aalderen, W. V., Life Without blood, *J. Cardiovasc. Surg.*, **1**, 133, 1960.
4) Churchill-Davidson, F., Sanger, C. and Thomlinson, R. H., High pressure oxygen and radiotherapy, *Lancet*, **1**, 1091, 1955.
5) Emery, E. W., Loucas, B. G. B. and Williams, K. G., Technique of irradiation of conscious patients under increased oxygen pressure, *Lancet*, **I**, 248, 1960.
6) Foster, C. A., Hyperbaric oxygen and radiotherapy, hyperbaric oxygenation, Edited by Ledingham I, McA. E. S. Livingstone LTD. 1965, 360 p.
7) Boerema, I., Influence of hyperbaric oxygen drenching whether or not combined with hyperthermia, hypothermia and cytostatics, in patients with cancer. Proceedings of the First International Congress. The clinical application of hyperbaric oxygen, 1964.
8) Kluft, O. and Boerema, I., Hyperbaric oxygen in experimental cancer in mice. Proceedings of the First International Congress. The clinical application of hyperbaric oxygen, 1964.
9) Adams, J. F. and Ledingham, I. M., The role of tissue hyperoxygenation in tumor chemotherapy. Proceedings of the First International Congress. The clinical application of hyperbaric oxygen, 1964.
10) Krementz, E. T. and Knudson, L., The effect of increased oxygen tension on the tumoricidal effect of nitrogen mustard, *Surgery*, **50**, 266, 1961.
11) Stern, K., Studies on reticulo-endothelial function in relation to groth process, *Ann. NY Acad, Sci.*, **88**, 1, 1960.
12) Herbut, P. A., Heterologus transplantation of human tumor, *Cancer Res.*, **16**, 408, 1956.
13) Old, L. J., Benacerraf, B., Clarke, D. A., Carswell, E. A. and Stockert, E., The role of the reticuloendothelial system in the host reaction to neoplasia, *Cancer Res.*, **21**, 1281, 1961.
14) Kiyono, K., Referat über die Leukozyten im Blut und im Gewebe, insbesondere über die histiozytären Zellen, *Verhand. Jap. Path. Gesell.*, 8, 1, 1918.
15) Aschoff, L., Das retikuloendoetheliale System, *Erg. Inn. Med.*, **26**, 1, 1964.

16) Akazaki, K., Reticulo-endothelial system, *SAISHIN-IGAKU*, **17**, 1018, 1962 (in Japanese).
17) Akazaki, K., Reticulo-endothelial system, *SAISHIN-IGAKU*, **16**, 36, 1961 (in Japanese).
18) Biozzi, G., Benaceraf, B. and Halpern, B. N., Quantitative study of the guranulopectic activity of the reticulo-endohtlial system, II: a study of the kinetics of the granulopectic activity of the R.E.S. in relation of the dose of carbon injected. relationship between the weight of the organs and their activity, *Brit. J. Exp. Path.*, **34**, 441, 1953.
19) Diluzio, N. R. and Wooles, W. R., Depression of phagocytic acitivity and immune response by methyl palmitate, *Amer. J. Physiol.*, **206**, 939, 1964.
20) Billingham, R. E., Brent, L. and Medawar, P. B., Actively acquired tolerance of foreign cells, *Nature*, **172**, 603, 1953.
21) Medawar, P. B., The behaviour and fate of skin autografts and skin homografts in rabbits, *J. Anat.*, **77**, 176, 1944.
22) Pace, D. M., Thompson, J. R. and Van Camp, W. A., Effects of oxygen on growth in several established cell lines, *J. Nat. Cancer Inst.*, **28**, 897, 1962.
23) Rueckert, R. R. and Mueller, G. C., Effect of oxygen tension on HeLa cell growth, *Cancer Res.*, **20**, 944, 1960.
24) Asano, T., Hattori, T., Mori, K., Osada, T., Okada, T., Takeichi, A. and Kito, T., The influence of oxygen at high pressure on the prolileration of cells in tissure culture, *Proc. Jap. Cancer Ass. 25th Ann. Meet.*, 1966 (in Japanese).
25) Mori, K., Study on adjuvant therapies in cancer chemotherapy, especially on the combination of hyperthermia and hyperbaric oxygenation, *Nagoya J. Med. Sci.*, **29**, 275, 1967.
26) Takeichi, A., Studies of the influence of oxygen at high pressure (OHP) on metastasis of Yoshida sarcoma, *J. Jap. Surg. Soc.*, **68**, 1968 (in Japanese), in Press.
27) Halpern, B. N., Reticuloendothelial structure and function, Ronald Press Company, 1960, 259 p.
28) Druckrey, H., Ergebnisse der experimentellen Krebstherapie, *Z. Krebsforsch.*, **47**, 112, 1938.
29) Nicol, T. and Bilbey, D. L., Substances depressing the phagocytic activity of the reticulo-endothelial system, *Nature*, **182**, 606, 1958.
30) Berg, J. W., Sinus histiocytosis fallacious measure of host resistance to cancer, *Cancer*, **9**, 935, 1956.
31) Black, M. M., Krepe, S. and Speer, F. D., Lymph node structure in patients with cancer of the breast, *Amer. J. Path.*, **29**, 505, 1953.
32) Berg, J. W., Inflamation and prognosis in breast cancer, A search for host resistance, *Cancer*, **12**, 714, 1959.
33) Yamagata, S., Miura, K., Kaneko, A. and Kikuta, Y., Measurement of reticulo-endothelial function, *SAISHIN-IGAKU*, **17**, 1065, 1962 (in Japanese).
34) Adler, H. and Reimann, F., Beitrag zur Funkionsprüfung des Reticuloendothelialen Apparates, *Z. Ges. Exp. Med.*, **47**, 617, 1925.
35) Ninomiya, Y., Uber die Methode zur Funktionsprüfung der Leber mittelst Kongorotes, *Tohoku J. Exp. Med.*, **11**, 151, 1928.
36) Ninomiya, Y., Die beziehungs des Reticuloendothelialsystems auf die Funktionsprüfung der Leber mit dem Kongorot, *Tohoku J. Exp. Med.*, **11**, 188, 1928.
37) Halpern, B. N., Benacerraf, B. and Biozzi, G., Quantitative study of the granulopectic activity of the reticulo-endothelial system, I: the effect of the ingredients present in india ink and substances affecting blood clotting *in vivo* on the fate of carbon particles administered intravenously in rats, mice and rabbits, *Brit. J. Exp. Path.*, **34**, 426, 1953.
38) Sasamoto, H., Nakayama, H. and Ota, Y., Hyperbaric oxygen therapy and oxygen toxicity, *Jap. Med. J.*, 2211, 9, 1966 (in Japanese).
39) Boerema, I., An operation room with high atomospheric pressure, *Surgery*, **49**, 291, 1961.
40) Tsuji, K., The experimental and clinical studies of the hypothermia on the chemo-

therapy of the malignant tumors, *J. Jap. Surg. Soc.*, **66**, 579, 1965 (in Japanese).
41) McFarlane, R. M. and Wermuth, R. E., The use of hyperbaric oxygen to prevent necrosis in experimental pedicle flaps and composite skin grafts, *Plast. Reconstr. Surg.*, **37**, 422, 1966.
42) Wharton, D. R. A., Miller, G. L., Whaston, M. L., Hankwitz, R. H. and Miller, E. E., The effect of tumors on antibody levels in mice, *Cancer Res.*, **11**, 127, 1951.
43) Parfentijev, I. A., Cliffton, E. E. and Duran-Reynals, F., Alteration of immunological response in malignancy: decline of proteus agglutirinin, *Science*, **113**, 523, 1951.
44) Fujinami, S., Study on anticancer power with special reference to the effect of liver X-ray irradiation upon the anticancer power, *J. Nagoya City Med. Ass.*, **17**, 567, 1967 (in Japanese).
45) Ozawa, M., Experimental studies on the influence of defencive power upon the homogeneous skin transplantation, *J. Nagoya City Med. Ass.*, **18**, 1968 in press (in Japanese).
46) Ishibashi, U., Prevention of recurrence of gastric cancer by immunity, *J. Jap. Surg. Soc.*, **65**, 883, 1954 (in Japanese).
47) Hanai, K., A study on allogeneic skin graft, the isolation of cell-free transplantation antigen with sephadex G-25, *Nagoya Med. J.*, **13**, 231, 1967.
48) Akiyama, T., Transplantation immunity, *KAGAKU*, **34**, 424, 1964 (in Japanese).
49) Ishibashi, U., Cancer and reticuloendothelial system, *Proc. Soc. Reticulcendothel. Systm*, **3**, 43, 1963 (in Japanese).
50) Fuji, G., Experimental study on the homograft, *Jap. J. Allerg.*, 8, 549, 1959 (in Japanese).