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RESPONSE OF A MUSCULO-SKELETAL TUMOUR TO COMBINED RADIATION THERAPY

An in vitro/vivo investigation

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Although it was first reported more than 60 years ago that elevated temperatures sensitised malignant tumours to irradiation, there has been little attempt to exploit this effect clinically or to carry out detailed investigations relating in vitro and in vivo tumour response. Similarly, a beneficial synergistic action between radiation therapy and cytotoxic drugs has been sought, but often the results have been obscured by imprecise monitoring of malignant cell response.

In 1910 MÜLLER combined irradiation and diathermy in the treatment of human cancer, and in 1913 he reported on 100 patients with advanced, histologically proven cancer of various sites (breast, bronchus, oesophagus and stomach); of these 100 tumours, 32 regressed completely and 36 temporarily. However, the effectiveness of the combined treatment was evaluated on the basis of personal experience rather than on a comparative series of tumours treated by each therapy alone. MÜLLER's publication was followed by several other reports, with a total consideration of 500 cases. The conditions of treatment in each series were however too variable and the information often insufficient to permit a valid comparison of the published results (ARONS & SOKOLOFF 1937).

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WARREN (1935) treated 32 cases of advanced carcinoma and sarcoma in man with a combination of induced fever of 41.5° C and irradiation. However, the remissions reported were usually of short duration (3 to 6 months). KORB (1939) showed a synergistic action between heat and irradiation on basal cell carcinomas, while WOEBER (1955) found that the effective radiation dose could be reduced in the presence of hyperthermia. CRILE (1962) reported that the dose required to induce a regression in cutaneous metastases from a breast carcinoma was reduced from 1 500 rad to 600 rad if the skin was immersed in a water-bath at 44 to 46° C for 1 hour. He also found that the squamous cell carcinoma, metastatic melanoma, and rectal carcinoma showed greater regression to radiation therapy after previous exposure to heat. BARTH (1956) found that there was more skin injury after combined heat and irradiation than with irradiation alone, findings which were at variance with the results of BIRKNER & WACHSMANN (1949) and WOEBER (1955).

ROHDENBURG & PRIME (1921) using the Crocker 180 tumour in mice and spontaneous mammary tumours of the Lathrop mouse strain, and HILL (1934) using a Jensen sarcoma and an unidentified mouse tumour, all found regression of the malignant growth when subjected to heat plus a sub-lethal dose of irradiation. ROHDENBURG & PRIME found no significant difference when either heat or radiation therapy was given first in combination therapy; while ARONS & SOKOLOFF (1937) and FUCHS (1935) reported that simultaneous therapy or the application of heat immediately subsequent to irradiation gave the best results. JARES & WARREN (1937) found that a time interval of twelve to twenty-four hours between heat and irradiation reduced the beneficial effects of the combined therapy. In a material of over 3 000 mice with Wood's sarcoma, the best results were obtained when hyperthermia was given first (49.5 per cent cure rate compared to 22.3 per cent).

The beneficial response of tumours to combined chemotherapy and radiation therapy has been reported in several series. MANUILA et coll. (1967) used a combination of nitrogen mustard and irradiation in Hodgkin's disease, while a synergistic effect between 5-fluorouracil and irradiation in pulmonary and oesophageal carcinoma has been reported (FOYE et coll. 1960, ALLAIRE et coll. 1961). The use of actinomycin D and irradiation in Wilms' tumour is another example of the adjuvant effect of combination therapy (FARBER et coll. 1960).

In the present investigation the adjuvant effect of non-curative (sub-optimal) irradiation in combination with a sub-optimal dose of local hyperthermia (tissue temperature above 42° C) or chemotherapy (Methotrexate) have been examined, and the results of in vitro measurements (respiration and anaerobic glycolysis) compared with the in vivo response (volumetric measurements and isotope uptake).

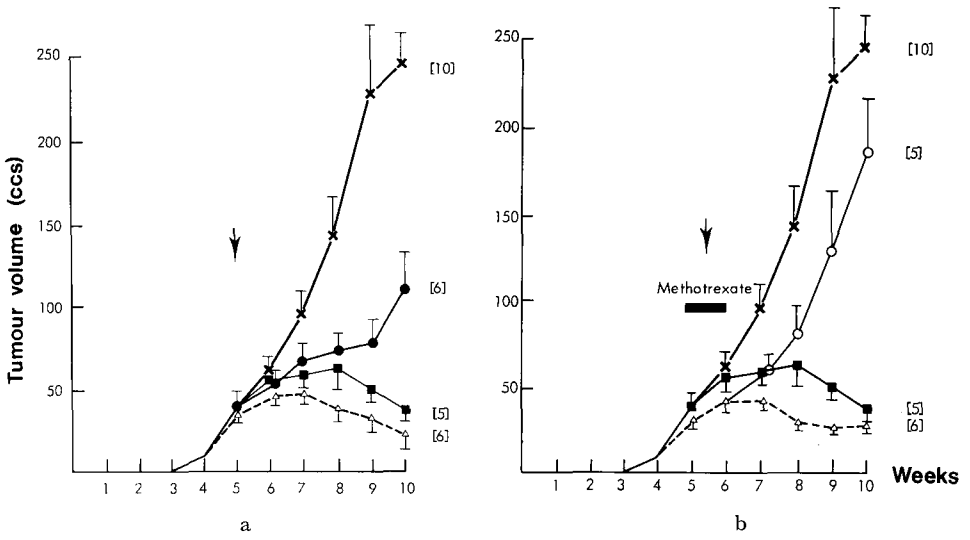

Fig. 1. Changes in mean Vx2 tumour volume following various combination therapies. The figures in brackets indicate the number of tumours (animals) at each point on the curves. a) × untreated tumours, ● hyperthermia, ■ radiation therapy, △ hyperthermia + radiation therapy. b) × untreated tumours, ○ methotrexate, ■ radiation therapy, △ radiation therapy + methotrexate.

Materials and Methods

The experimental musculo-skeletal tumour used was the Vx2 tumour, a highly malignant growth which originated in a Shope virus-papilloma. The tumour is characterized by rapid and predictable metastases to lymph nodes and lungs. The tumour was passaged by periodic transfer of 1×10^6 cells into the thigh muscles of New Zealand white rabbits, and the resulting tumour became palpable between 3 and 4 weeks later, the volume increased exponentially from 5 to 9 weeks; untreated rabbits died at 10 weeks. Therapy was applied at day 35 after cell inoculation and tumour volume measurements were made at weekly intervals, allowances being made for the normal tissues of the thigh.

For the in vitro experiments cells were obtained from treated and control tumours by enzymatic disaggregation with trypsin and DNase (Muckle & Dickson 1971) which does not injure cell metabolism.

The O_2 uptake (cell respiration) and CO_2 production (anaerobic glycolysis) were measured in Warburg flasks, with 5 to 10×10^6 cells per flask, incubated at 37.5° C. (For details see Muckle & Dickson.)

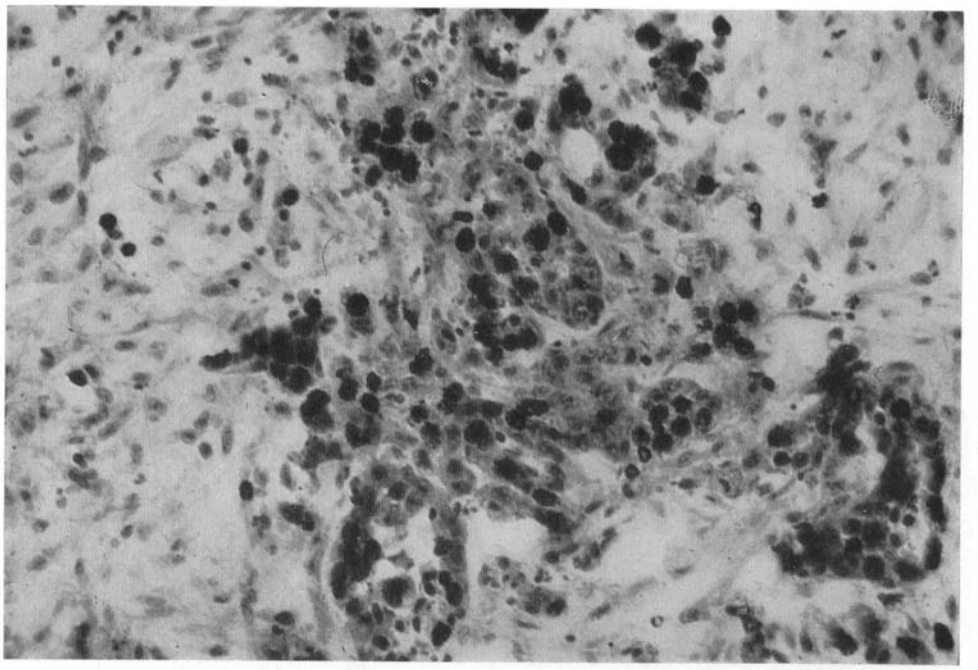

Fig. 2. Untreated Vx2 tumour with ^{3}H-thymidine uptake in groups of cells in a fibrous tissue stroma (×410).

For the in vivo treatment of tumours by heat or irradiation the rabbit was anaesthetized with intravenous Nembutal, 0.6 ml/kg. Local hyperthermia was applied by immersion of the hind limb in a water-bath at 46° C; when an intratumour temperature of 42 to 43° C was reached, and maintained for 1 hour. Needle electrodes measured tumour, thoracic, skin (ear) and abdominal temperatures.

Radiation therapy, 1 000 rad TD, was applied by superficial radiation (140 kV, 8 mA, filtration 0.2 mm Cu and 1.0 mm Al) from 2 opposing fields, size 7 cm, FSD 25 cm. Chemotherapy consisted of methotrexate given by 6 daily intravenous injections of 0.4 mg/kg into the ear vein, beginning on day 35 after tumour inoculation. In combination therapy, irradiation was performed on day 38 or followed within 2 hours of hyperthermia.

For isotope examinations 100 μCi ^{3}H-thymidine (thymidine methyl T, greater than 10 000 mCi/mM, Amersham) were injected into the femoral artery of the tumour bearing limb. Biopsies were taken at regular intervals. When combination therapy was given, the administration of the isotope followed immediately after the treatment ceased.

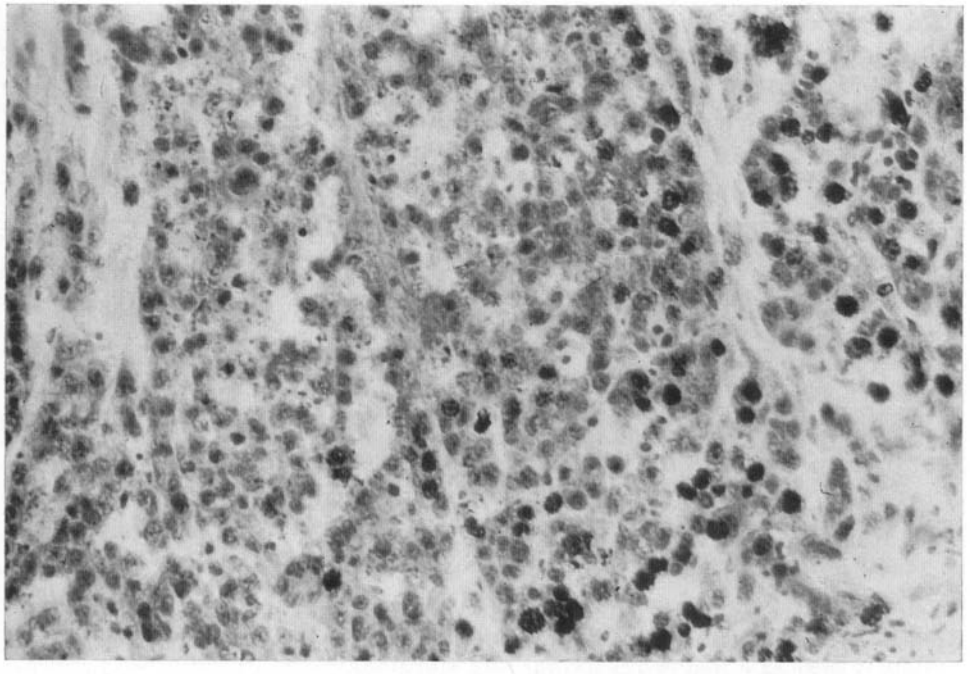

Fig. 3. Treated Vx2 tumour (heat and irradiation) reduced ^{3}H-thymidine 5 days after therapy. Lysis and karyorrhexis in the Vx2 cells ($\times 410$).

Results

Fig. 1 records the response of the primary tumour to hyperthermia and irradiation, alone or in combination, and chemotherapy and irradiation, alone or in combination. Each method, when applied individually, resulted in a restraint of tumour growth compared to the untreated controls; the difference in volume of the control and treated tumours was significant at the 8th week ($p < 0.001$). However, the tumours treated by heat or methotrexate subsequently increased in volume, whereas the irradiated tumours decreased in size. Following combination therapy, tumour volume continued to increase for a further 2 weeks. Thereafter, the tumours given radiation therapy plus hyperthermia or plus methotrexate, decreased in volume, the tumour volume measurements for both types of dual therapy being comparable at each time point ($p > 0.05$), and the variability about the mean values being small. The volumes of these tumours were significantly less than the corresponding volumes for tumours irradiated only ($p < 0.05$).

Table 1

Each Warburg flask contained 5–10 × 10^6 Vx 2 cells. The respiration (QO_2) and anaerobic glycolysis, (QCO_2) (in brackets) of V × 2 tumour cells following in vivo therapy. The figures quoted as a range were obtained from 3 separate tumours in each case. Q values represent μl gas exchanged/mg dry weight of cells/per hour.

Therapy	24 hours	10 days	4 weeks
Irradiation (QO_2)	4.0–4.7	7.3–8.0	7.0–8.0
Irradiation (QCO_2)	(8.0–8.8)	(12.6–13.8)	(14.0–15.0)
Heat (42° C), 1 hour	2.5–3.3	4.5–5.6	6.5–8.1
	(14.2–16.4)	(14.0–16.7)	(14.1–17.7)
Methotrexate	9.8–10.6	4.8–4.9	7.6–7.7
	(14.4–15.6)	(8.8–10.0)	(14.0–16.0)
Untreated cells	7.7–9.2	7.5–9.2	7.3–8.9
	(13.9–17.1)	(14.0–17.2)	(13.8–16.9)
Heat/irradiation	3.3–4.0	4.1–5.2	4.0–4.7
	(7.0–8.1)	(9.0–10.0)	(14.5–16.3)
Irradiation/methotrexate	4.8–5.3	4.4–5.5	5.3–5.6
	(8.6–9.2)	(8.8–9.8)	(12.2–13.6)

Fig. 2 depicts the normal histology of the Vx2 muscle tumour, and the isotope labelling is seen within the nests of Vx2 cells. The changes after hyperthermia and irradiation consist of loss of the normal tumour architecture, with cell lysis (Fig. 3). There is a peripheral rim of intact cells with isotope uptake. Viable Vx2 cells could be obtained from this area even when the central part was replaced by fibrous tissue 4 weeks after therapy. The potentiating effect of heat or methotrexate produced no striking alteration in the histologic picture following irradiation.

The Table records the respiration and anaerobic glycolysis of the treated tumours at 24 hours, 10 days and 4 weeks following therapy. Oxygen uptake and CO_2 production were linear over 4 hours in Warburg flasks, and for comparison the average QO_2 and QCO_2 values over this period have been used. Local heating for 1 hour caused a rapid decrease in respiration, with subsequent recovery of QO_2 values, while anaerobic glycolysis was unaffected. However, irradiation had a depressive effect on both respiration and glycolysis, measured 24 hours after therapy, but both these parameters recovered towards control values over the following 4 weeks. The effect of methotrexate was evident at 10 days as an inhibition of both O_2 uptake and CO_2 production; at 4 weeks these values returned to normal. The effects of combination therapy reflected a sum-

mation of the individual therapies; at 4 weeks there was synergism of action between irradiation and hyperthermia or methotrexate, indicated by persistent marked inhibition of respiration.

The effects of combination therapy and each therapy alone on tumour volume, histology and isotope uptake (in vivo) were comparable to the effects on O_2 uptake, and to a lesser extent the CO_2 production (in vitro), after each form of treatment.

Discussion

Experiments on the Vx2 musculo-skeletal tumour showed that the effects of irradiation were potentiated by heat or chemotherapy (Methotrexate) as assessed by a significant reduction in tumour volume (Fig. 1) and the inhibition of respiration and anaerobic glycolysis (Table). Although the insensitivity of tumour growth measurements as the sole criterion in the assessment of cancer therapy is well recognized (STEEL & LAMERTON 1969), the effects of combination therapy were also shown by the marked reduction in radioactive ^{3}H-thymidine uptake in treated tumours, and the presence of cell lysis and karyorrhexis. However, the biochemical and histologic results indicated that after combination therapy considerable numbers of metabolically and mitotically active Vx2 cells remained in the primary tumour and the animal survival data corroborated these findings, combination therapy having little effect on host death (74 ± 5 days in the treated rabbits compared to 70 ± 6 days in the controls). Vx2 tumour regression has been observed after combined radiation therapy and chemotherapy (MORRISON & JOHNSTON 1968) with a significantly increased host survival time. FUCHS (1935) described similar histologic changes after combined heat and radiation therapy as found in the above experiments.

CRILE (1970) has drawn attention to the possibility that a primary tumour may act as the antigenic source necessary to maintain immunity against metastases. With tumour systems in mice the incidence of secondary growths was significantly increased when the foot carrying the tumour was amputated as when the tumour was destroyed by irradiation. CRILE suggested that the cells killed by irradiation maintained their antigenicity and prolonged the animals' immunity.

Elevation of temperature results in vasodilatation and increased tissue oxygen tension, and it has been reported that an increase in oxygen tension has a tumouricidal effect (KLUFT & BOEREMA 1963) and potentiates the effect of irradiation (WILDERMUTH 1964). From the present work it is also clear that the relationship between primary tumour and metastases requires consideration in therapy. It may be that irradiation can be enhanced by prior heat application, as suggested

by many authors in the earlier part of this century, and that the effects of elevated temperatures on cancer cell metabolism (disruption of cell membranes, arrest of mitotic division in metaphase, and prolongation of the lag phase with inhibition of the log phase of cell growth; for details see MUCKLE & DICKSON 1971) will sensitise the neoplasm to irradiation. Total body hyperthermia techniques as advocated by HENDERSON & PETTIGREW (1971), may be of value in future combined therapy in human cancer.

The value of the present in vivo/vitro investigation was the demonstration of biochemical changes paralleling tumour growth measurements. Such techniques could be of use in human therapy although the difficulty in obtaining cancer cells from solid neoplasms is well recognized; and a complex, pains-taking procedure was needed to obtain viable Vx2 cells from the established tumour (MUCKLE & DICKSON 1971, MUCKLE 1972). However, such advances in cancer physiology will be necessary to monitor the response of malignant cells to radiation therapy, either alone or in combination with heat or chemotherapy.

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SUMMARY

An experimental musculo-skeletal tumour, the Vx2 tumour in rabbits, proved to be a convenient model to investigate the effects of irradiation, either alone or in combination with heat or chemotherapy. In vitro (cell respiration and glycolysis) and in vivo (tumour growth, isotope uptake) measurements were carried out on treated and untreated tumours. Combination therapy proved to be more effective on all parameters than either therapy alone, and the in vitro response of tumour cells after treatment paralleled the in vivo response. The implications of combination therapy in man is discussed, with a view to sensitising cancer cells to irradiation and potentiating the immune response.

ZUSAMMENFASSUNG

Ein experimenteller Muskel-Skelett-Tumor, der Vx2 Tumor bei Kaninchen, wurde als ein geeignetes Modell befunden, um die Effekte von Strahlung, entweder alleine oder in Kombination mit Wärme oder Chemotherapie zu untersuchen. In vitro (Zell-Atmung und Glykolysis) und in vivo (Tumorwachstum, Isotopaufnahme) Untersuchungen wurden an behandelten und nicht behandelten Tumoren vorgenommen. Die Kombinationstherapie erwies sich bei allen Parametern als stärker wirksam als jegliche Behandlung für sich, und die in vitro Reaktion der Tumorzellen nach einer Behandlung folgte der in vivo Reaktion. Die Folgerungen einer kombinierten Therapie beim Menschen im Hinblick auf die Sensibilisierung von Krebszellen gegen Bestrahlung und die Verstärkung der Immunantwort werden diskutiert.

RÉSUMÉ

Une tumeur musculo-squelettique expérimentale, la tumeur Vx2 des lapins, s'est montrée être un modèle convenable pour étudier les effets de l'irradiation soit seule, soit associée à la chaleur ou à la chimiothérapie. Les mesures in vitro (respiration cellulaire et glycolyse) et in vivo (croissance tumorale, fixation isotopique) ont été effectuées sur des tumeurs traitées et non traitées. L'association thérapeutique s'est montré plus efficace sur tous les paramètres qu'aucun traitement isolé et la réponse des tumeurs cellulaires après traitement in vitro est parallèle à la réponse in vivo. L'auteur examine les conséquences de cette association thérapeutique chez l'homme dans le dessein de sensibiliser les cellules cancéreuses à l'irradiation et de potentialiser la réponse immunitaire.

REFERENCES

ALLAIRE F. J., THIEME E. T.and KORST D. R.: Cancer chemotherapy with 5-fluorouracil alone and in combination with X-ray therapy. Cancer Chemother. Abstr. 14 (1961), 59.

ARONS I. and SOKOLOFF B.: Combined roentgentherapy and ultrasound. Amer. J. Surg. 36 (1937), 533.

BARTH G.: Radiology Book Abstract, p. 172. 1956.

BIRKNER R. und WACHSMANN F.: Über die Kombination von Röntgenstrahlen und Kurzwellen. Strahlentherapie 76 (1949), 93.

CRILE G.: Selective destruction of cancers after exposure to heat. Ann. Surg. 156 (1962), 404.

— Criticism of conventional methods of treating solid tumours in man. Brit. med. J. 4 (1970), 489.

FARBER S., D'ANGIO G., EVANS A. and MITUS A.: Clinical studies on actinomycin D with special reference to Wilms' tumour in children. Ann. N.Y. Acad. Sci. 89 (1960), 421.

FOYE L. V., WILLET F. M., HALL B. and ROTH M.: The proliferation of radiation effects with 5-fluorouracil. Cancer Chemother. Abstr. 6 (1960), 12.

FUCHS H. T.: Maligne Zelle und Embryonalzelle. Klin. Wschr. 14 (1935), 1295.

HENDERSON M. A. and PETTIGREW R. T.: Induction of controlled hyperthermia in treatment of cancer. Lancet (1971), 1275.

HILL L.: Action of ultrashort waves on tumours. Brit. med. J. 2 (1934), 370.

JARES J. J. and WARREN S. L.: Cancer Problem Symposium, p. 225. 1937.

KLUFT O. and BOEREMA I.: Hyperbaric oxygen in experimental cancer in mice. Clinical application of hyperbaric oxygen. Proc. 1st. International Congress, Amsterdam, p. 126. 1963.

KORB H.: Vergleichende Temperaturmessungen am Menschen bei den Wellenlängen 6 m und 1 m. Strahlentherapie 64 (1939), 536.

MANUILA L., MODES S. and RENTCHNICK P.: *In:* New trends in the treatment of cancer. Springer-Verlag, Berlin 1967.

MUCKLE D. S.: The effect of hyperthermia on the growth and metabolism of the Vx2 carcinoma. M. S. Thesis. University of Newcastle. 1972.

— and DICKSON J. A.: The selective inhibitory effect of hyperthermia on the metabolism and growth of malignant cells. Britt. J. Cancer. XXV (1971), 771.

MÜLLER C.: Die Röntgenstrahlenbehandlung der Malignen Tumoren und ihre Kombinationen. Strahlentherapie iii (1913), 177.

MORRISON J. M. and JOHNSTON I. D. A.: Effect of combined I. A. Methotrexate and irradiation on the growth rate of the Vx2 carcinoma in rabbits. Brit. J. Surg. 55 (1968), 395.

ROHDENBURG G. L. and PRIME F.: Effect of combination radiation and heat on neoplasms. Arch. Surg. 2 (1921), 116.

STEEL G. G. and LAMERTON L. F.: Cell population kinetics and chemotherapy. *In:* Human tumour cell kinetics. Nat. Cancer Inst. Monogr. 30 (1969), 29.

WARREN S. L.: Preliminary studies of the effect of artificial fever upon hopeless tumour cases. Amer. J. Roentgenol. 33 (1935), 75.

WILDERMUTH O.: Hyperbaric radiation therapy in cancer management. Radiology 82 (1964), 767.

WOEBER K.: Combination X-ray and ultrasound treatment for dermatological conditions biological foundations and clinical application. Amer. J. Phys. Med. 34 (1955), 376.