Br. J. Cancer (1980) **41**, Suppl. IV, 64

FEATURES AND LIMITATIONS OF THE *IN VIVO* EVALUATION OF TUMOUR RESPONSE BY OPTICAL MEANS

H. S. REINHOLD AND A. V.D. BERG-BLOK

From the Radiobiological Institute TNO, Rijswijk and Erasmus University, Rotterdam, The Netherlands

Summary.—When a tumour is growing in a thin sheet-like fashion, many useful parameters can be directly determined by optical means. In a "sandwich" system a tumour grows in a thin, separated layer of subcutis on the back of a rodent. The tumour itself is enclosed between two transparent surfaces, one being a glass cover slip. Living tumour cells appear to be relatively transparent; therefore, the blood vessels in the tumour are the most outstanding structures. The assay methods that can be applied for determining tumour response can be divided into three groups: (1) observation and recording of the nature of the tumour vasculature, etc.; (2) dynamic investigations on blood flow; and (3) the use of optical indicators for a relative index of tissue oxygenation state. Especially with regard to the latter methods, recent developments are promising. However, as determinations with these systems do not reveal the clonogenic viability of tumour cells as such, the use of such "observation" systems should be of necessity restricted to answering questions about tumour response that cannot be evaluated by other means.

MANY factors are involved in the ways tumour cells can be inactivated by treatment and thus the ultimate response to treatment will depend on the interaction of several variables. For radiation therapy, the most important one seems to be the proportion of hypoxic cells in tumours. The existence of hypoxic cells was predicted by Thomlinson & Gray in 1955 on the basis of histological findings in combination with mathematical models of oxygen diffusion. During the last two decades, hypoxic cells have almost invariably been found by means of bioassays in experimental tumours. The proportion of hypoxic cells in untreated experimental tumours is typically between 10% and 20% of the clonogenic cell mass. In view of its importance for the radiosensitivity of tumours, information on the mechanisms giving rise to tumour-cell hypoxia should be available. As tumour-cell hypoxia is obviously a phenomenon that is physiological, it should be studied by physiological means. This is especially important, as methods to perform bioassays on human tumours for the determination of hypoxic cells will be difficult to establish.

To obtain information on factors that are of influence in the distribution of oxygen in tumours, several systems have been developed on the basis of Algire's (1943) observation chamber. Merwin *et al.* (1950), Yamaura *et al.* (1971), Yamaura & Matsuzawa (1979), Reinhold (1971) and Reinhold *et al.* (1979) have adapted this method for use in radiobiological investigations. They have improved the observation chamber technique in such a way that, after inoculation, the tumour is allowed to grow only in a sheet-like fashion. This is generally done by having the tumour grow in an isolated sheet of subcutis, which is enclosed between two narrowly spaced transparent surfaces (Fig. 1). Similar systems for the hamster cheek pouch were designed by Warren *et al.* (1978). It should be emphasized that, to obtain reliable information from such observation chambers, it is absolutely necessary to have the tumour growing in a thin sheet. The reason is that, for a thickness of 100–200 μm, the tumour tissue is

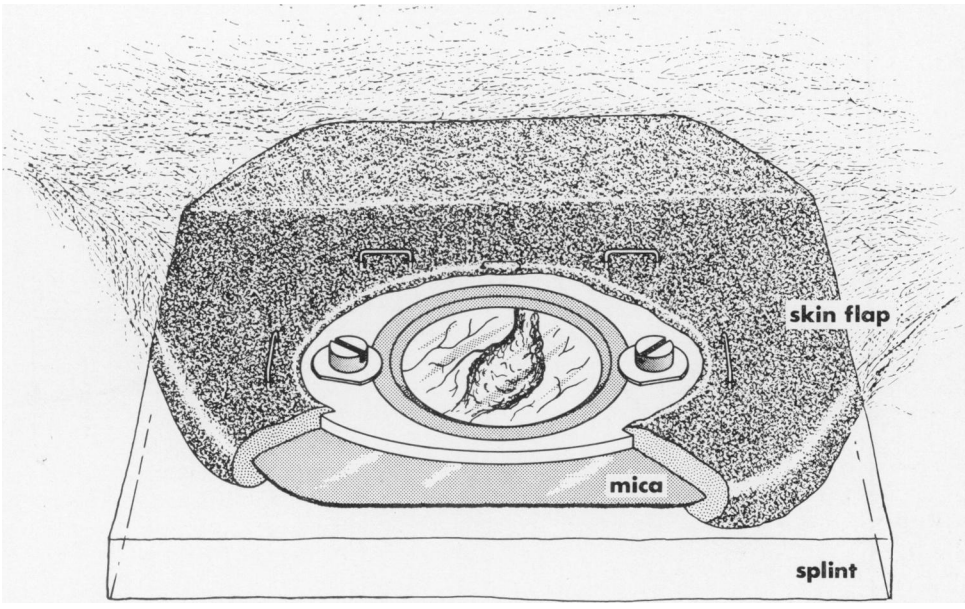

FIG. 1.—The "sandwich" tumour system. The tumour is implanted in a sheet of subcutis and is enclosed between a glass coverslip and a sheet of mica. Thickness is limited to 100–200 μm. Maximum diameter is about 8 mm.

sufficiently transparent for detailed observation and optical measurements; if the tumour exceeds this thickness, the picture becomes blurred. This not only severely restricts any measurements but it also becomes impossible to judge where supplying vessels come from. The idea behind the "sandwich" type of observation chambers is to perform determinations in what is essentially an equatorial cross section through a tumour. However, one has always to keep in mind that even the most sophisticated "sandwich" preparation still has a thickness of between 50 and 200 μm. This is 4 to 12 cell layers thick.

Viable tumour tissue in these preparations appears surprisingly transparent and clear (Algire, 1943; Kligerman & Henel, 1961). Blood vessels are the most outstanding structures and necrotic areas can be easily distinguished as "cloudy" regions. This is probably due to a change in the refractive index when a tissue area becomes necrotic. It is therefore a simple matter to obtain photomicrographs in which the tumour size, its vascular pattern and, when present, the necrotic areas can be recorded and followed for a number of days (Yamaura & Matsuzawa, 1979) with quantitative analysis.

However, some of the questions one would like to answer with systems like the one described are: Why, when and where are the hypoxic cells located in these tumours? How do they respond to therapy? Recent developments in methods for the optical analysis of metabolic processes as well as highly sophisticated methods for quantitative determinations on the microcirculation will no doubt yield a large body of information regarding tumour physiology that can be used to gain insight into the processes in human tumours. In addition to the aforementioned photographic recording of the vascular pattern and necrosis with their quantitative evaluation, the quantitative application of fluorescence microangiography (Reinhold, 1971), the topical determination of the local redox state (Gosalvez *et al.*, 1972; Reinhold *et al.*, 1979) and the topical analysis of erythrocyte velocity and regional tumour blood flow are all possible (Endrich *et al.*, 1979*b*).

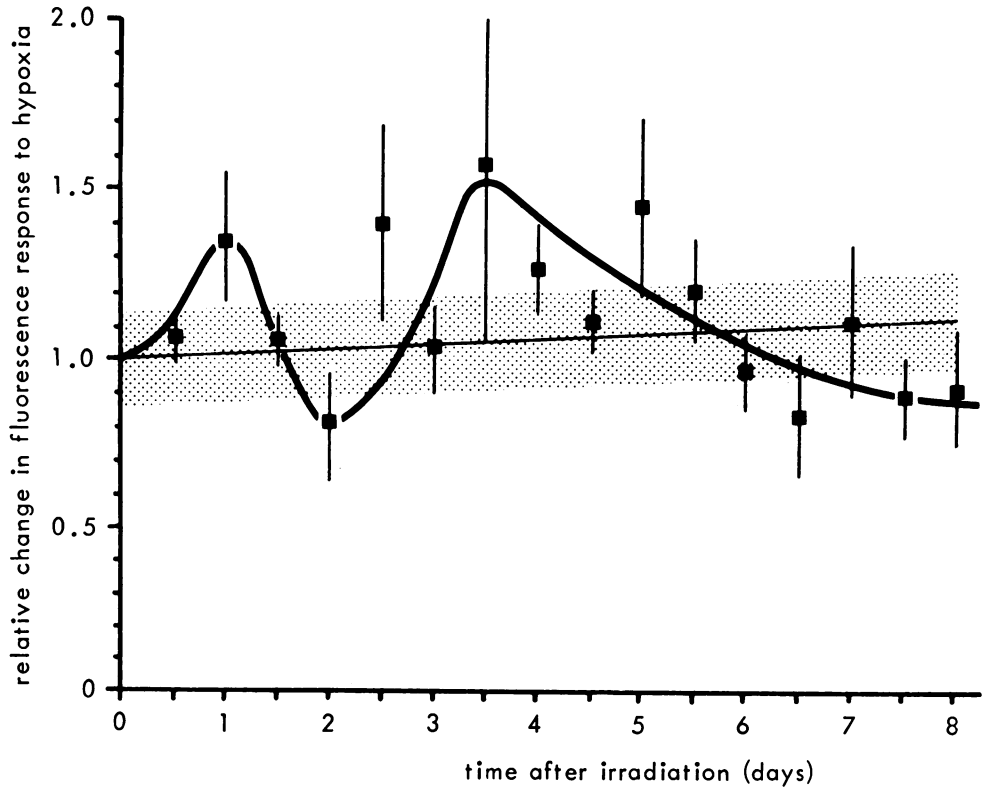

FIG. 2.—The hypoxic NAD(H) response as an indicator for tumour reoxygenation at various times after a dose of 20 Gy. Shaded area indicates s.e. mean of unirradiated (control) tumours.

To the exciting future applications belongs the distribution of oxygen assayed by fluorescent probes like pyrene butyric acid. Mitnick & Jobsis (1976) have used this indicator for determination of the oxygen tension in brain. The recently developed optical indicators for tissue pH (Lubbers *et al.*, 1977; Visser *et al.*, 1979) might also be adapted for use in "sandwich" tumour systems. This would be of great importance for investigations on the response of tumours to hyperthermia. In addition, it must be possible to estimate the rate of diffusion of chemotherapeutic agents with fluorescent properties, such as adriamycin, into the tumour tissue. Finally, the determination of the oxygenation state of the blood by absorption spectrometry (Vaupel *et al.*, 1978) seems to be within the range of possibilities.

Some, but not all, of the aforementioned methods have been used for the determination of tumour response to irradiation, none, to the authors' knowledge, to chemotherapy and only two methods for hyperthermia. Merwin *et al.* (1950) observed vascular changes in the original Algire-type of observation chamber, while Yamaura & Matsuzawa (1979) more recently pointed to the unexpected occurrence of regrowth foci after irradiation from the periphery of the tumour rather than the (hypoxic) centre. Reinhold (1971) has followed the microcirculation in tumours by means of microangiography and found an improvement in the circulation during fractionated radiation therapy. The oxygenation state of the tissue also responded with an improvement, followed by a dip and a subsequent slight recovery (Fig 2) (Reinhold *et al.*, 1979), after a dose of 20 Gy to "sandwich" tumours in the

rat. This pattern was almost identical to the one described by Thomlinson (1969) with a regrowth delay method. A decrease in blood velocity in a tumour during hyperthermia was found by Endrich *et al.* (1979*a*). This supported the finding of Reinhold *et al.* (1977) that stoppage of the microcirculation in the central parts of "sandwich" tumours may take place after prolonged hyperthermic exposure.

"Sandwich" tumour preparations are laborious to make and require a high degree of skill for making the preparations as well as for performing the determinations. Moreover, the preparations are fragile; this means that the risk of losing animals, *e.g.* during anaesthesia, depends greatly on the experimental procedure. The majority of the determinations, such as velocity measurements or NAD(H) redox determinations, are also time-consuming. As a result, the number of animals as well as the number of determinations that can be performed are necessarily restricted. On the other hand, only with these kind of microphysiological methods can effects such as the occurrence of fluctuation in blood supply (Intaglietta *et al.*, 1977) or NAD(H) fluorescence (Reinhold *et al.*, 1977) be demonstrated.

Obviously, one has to balance the efforts expended to perform a limited number of detailed physiological determinations against the more easily obtainable data from bioassays or histology. While gain in the quality of information obtained with bioassays may be achieved by using *in vivo/in vitro* methods (Barendsen, this symposium), gain in the quality of the optical determinations will have to come from better application and detection of, *e.g.* fluorescent probes, as well as from improved data acquisition and handling. For example, "sandwich" tumours are presently being analysed by single-line scanning for oxygenation (pyrene butyric acid fluorescence quenching) as well as for the NAD(H) hypoxic response. This means in practice that two different parameters are analysed at the same site, the numbers of sites per tumour ranging from 200 to 400. These numbers allow the construction of histograms for individual tumours for hypoxic NAD(H) response as well as, hopefully, values for the tissue oxygenation. The application of the linearity of the latter is presently being scrutinized (Lubbers & Opitz, 1976). Generally speaking, there must be possibilities to perform many of the aforementioned determinations at the same time in the same tumour, generating a two-dimensional image of a great number of factors concerned with the response of tumour microphysiology to treatment.

REFERENCES

ALGIRE, G. H. (1943) An adaptation of the transparent-chamber technique to the mouse. *J. Natl Cancer Inst.*, **4**, 1.

ENDRICH, B., ZWEIFACH, B. W., INTAGLIETTA, M. & REINHOLD, H. S. (1979*a*) Influence of temperature on microvascular hemodynamics during the early growth of the BA 1112 rat sarcoma. In *Microvascular Research; Abstracts of the Second World Congress for Microcirculation*, **17**, S120.

ENDRICH, B., REINHOLD, H. S., GROSS, J. F. & INTAGLIETTA, M. (1979*b*) Tissue perfusion inhomogeneity during early tumour growth in rats. *J. Natl Cancer Inst.*, **62**, 387.

GOSALVEZ, M., THURMAN, R. G., CHANCE, B. & REINHOLD, H. S. (1972) Indication of hypoxic areas in tumours from *in vivo* NADH fluorescence. *Eur. J. Cancer*, **8**, 267.

INTAGLIETTA, M., MYERS, R. R., GROSS, J. F. & REINHOLD, H. S. (1977) Dynamics of microvascular flow in implanted mouse mammary tumours. In *Proc. 9th Eur. Conf. Microcirculation. Antwerp* 1976. *Bibl. Anat.*, **15**, 273. Basel: Karger.

KLIGERMAN, M. M. & HENEL, D. K. (1961) Some aspects of the microcirculation of a transplantable experimental tumor. *Radiology*, **76**, 810.

LUBBERS, D. W. & OPITZ, N. (1976) Quantitative fluorescence photometry with biological fluids and gases. In *Oxygen Transport to Tissue II*. Ed. J. Grote, D. Reneau & G. Thews. New York: Plenum Publishing Corporation.

LUBBERS, D. W., OPITZ, N., SPEISER, P. P. & BISSON, H. J. (1977) Nano-encapsulated fluorescence indicator molecules measuring pH and pO_2 down to submicroscopical regions on the basis of the optode-principle. *Z. Naturforsch.*, **32c**, 133.

MERWIN, R., ALGIRE, G. H. & KAPLAN, H. S. (1950) Transparent-chamber observations of the response of a transplantable mouse mammary tumour to local röntgen irradiation. *J. Natl Cancer Inst.*, **11**, 593.

MITNICK, M. H. & JOBSIS, F. F. (1976) Pyrene-butyric acid as an optical oxygen probe in the intact cerebral cortex. *J. Appl. Physiol.*, **41**, 593.

REINHOLD, H. S. (1971) Improved microcirculation in irradiated tumours. *Eur. J. Cancer*, **7**, 273.

REINHOLD, H. S., BLACHIEWICZ, B. & BERG-BLOK, A. (1977) Oxygenation and reoxygenation in "sandwich" tumours. In *Proc. 9th Eur. Conf.*

Microcirculation. Antwerp. Bibl. Anat., **15,** 270. Basel: Karger.

REINHOLD, H. S., BLACHIEWICZ, B. & BERG-BLOK, A. (1979) Reoxygenation of tumours in "sandwich" chambers. *Eur. J. Cancer*, **15,** 481.

THOMLINSON, R. H. & GRAY, L. H. (1955) The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br. J. Cancer*, **9,** 539.

THOMLINSON, R. H. (1969) Reoxygenation as a function of tumor size and histopathological type. In *Proc. Carmel Symposium on Time and Dose Relationships in Radiation Biology as Applied to Radiotherapy*. Carmel, 1969. BNL 50203 (C-57), p. 242.

VAUPEL, P., GRUNEWALD, W. A., MANZ, R. & SOWA, W. (1978) Intracapillary HbO_2 saturation in tumor tissue of ds-carnosarcoma during normoxia. In *Oxygen Transport to Tissue II*. Eds I. A. Silver, M. Erecinska & H. I. Bicher. New York: Plenum Publishing Corporation.

VISSER, J. W. M., JONGELING, A. A. M. & TANKE, H. J. (1979) Intracellular pH-determination by fluorescence measurements. *J. Histochem. Cytochem.*, **27,** 32.

WARREN, B. A., SHUBIK, P., WILSON, R., GARCIA, H. & FELDMAN, R. (1978) The microcirculation in two transplantable melanomas of the hamster. I. *In vivo* observations in transparent chambers. *Cancer Lett.*, **4,** 109.

YAMAURA, H., SUZUKI, M. & SATO, H. (1971) Transparent chamber in the rat skin for studies on microcirculation in cancer tissue. *Gann*, **62,** 177.

YAMAURA, H. & MATSUZAWA, T. (1979) Tumour regrowth after irradiation. An experimental approach. *Int. J. Radiat. Biol.*, **35,** 201.