Br. J. Cancer (1980) **41**, Suppl. IV, 245

INFLUENCES OF THE HOST AND LOCAL CONDITIONS ON THE *IN VIVO* CLONOGENIC EXPRESSION OF SUBCUTANEOUSLY INOCULATED R-1,M TUMOUR CELLS

A. F. HERMENS

From the Radiobiological Institute of the Organisation for Health Research TNO, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands

Summary.—In order to study possible variations in the expression of the clonogenic capacity of cultured R-1,M tumour cells due to different conditions of the growth substrate, assays were performed by employing the *in vitro* plating technique described by Puck & Marcus (1956) and the *in vivo* TD_{50} assay developed by Hewitt & Wilson (1959). Assays were performed with cell suspensions containing R-1,M cells alone or admixed with either heavily irradiated R-1,M cells designated as F(R-1,M) cells or normal, syngeneic MER-1 cells that have a phagocytic capacity.

***In vitro* assays demonstrated a maximal capacity for colony formation of 80 to 100% of the R-1,M cells plated. TD_{50} assays performed with the syngeneic WAG/Rij rat and the allogeneic BALB/c.nu mouse revealed that R-1,M cells can express their clonogenic capacity in both strains equally well, with a TD_{50} of 6000 cells.**

From results of assays performed with admixed cells, it was concluded that, in the BALB/c.nu mouse, MER-1 cells are capable of reducing the TD_{50} by a factor of 600, while admixture with both MER-1 and F(R-1,M) cells in the WAG/Rij rat resulted in a reduction by a factor of only 3–4.

The intrinsic radiosensitivity of R-1,M cells grown in single cultures, and mixed cultures with MER-1 cells, was studied by the *in vitro* assay after *in vitro* irradiation. For R-1,M cells D_Q and D_0 values of 2·5 and 1·3 Gy, respectively, were obtained. However, *in vivo* assays for survival of *in vitro* irradiated R-1,M cells in single culture provided data which cannot be correlated in a simple manner with data obtained by the *in vitro* assay.

ONE elementary requirement for obtaining reproducible and interpretable results with any experimental tumour system is that the assay system is stable and provides conditions for the optimal expression of the clonogenic capacity of the surviving clonogenic cells. With some types of tumour lines, the employment of the *in vivo* TD_{50} assay developed by Hewitt & Wilson (1959) results in a 50% tumour incidence with less than 10 cells. However, in other tumours the TD_{50} is in the order of 1000 cells. Furthermore, *in vivo* assays of tumours irradiated *in vivo* are complicated by many factors, especially after fractionated treatment (Hermens & Barendsen 1969; 1977; Reinhold 1974).

Since there is a demand for more direct information on responses of primary tumours, especially of human origin, the immune deficient mouse, such as the nude BALB/c strain, has been introduced for xenograft studies.

In order to obtain more information about dose response relationships obtainable with different systems, experiments were performed with R-1,M tumour cells irradiated *in vitro* and assayed *in vitro* or *in vivo* using syngeneic and allogeneic animals. The results obtained with these experiments show that the expression of the clonogenic capacity of the R-1,M cells is influenced significantly by both local conditions and the assay system employed. Furthermore, the dose response relationship obtained by *in vivo* assay is not always a simple reflection of the intrinsic radio-sensitivity as measured *in vitro*.

Essentially, 2 series of experiments were performed. One was designed to measure the number of cultured R-1,M

tumour cells needed for 50% tumour takes (TD_{50}) upon transplantation into the syngeneic WAG/Rij rat or the allogeneic BALB/c.nu nude mouse. Furthermore, effects from an altered local milieu were investigated by admixing the tumour cells with either heavily irradiated tumour "feeder" cells, F(R-1,M), or with normal syngeneic MER-1 cells which show a phagocytic capacity *in vitro*. Dose response relationships were measured for R-1,M tumour cells irradiated *in vitro* and assayed by the *in vitro* plating technique (Puck & Marcus, 1956) or the *in vivo* TD_{50} assay (Hewitt & Wilson, 1959). For the latter assay, both syngeneic and allogeneic strains of animals were used.

MATERIALS AND METHODS

Cell lines.—The R-1,M tumour cell line was obtained by subcloning the R-1 cell line in 1972 from the BA1112 rat rhabdomyosarcoma described by Barendsen & Broerse (1969). Each of the tumour cell lines, *i.e.* the cells isolated from the BA1112 rhabdomyosarcoma system (Reinhold, 1965), and the R-1 and R-1,M cells, give rise to transplantable tumours upon inoculation into the syngeneic inbred strain of WAG/Rij rat. Over a period of 5 years, no major differences have been demonstrated between the *in vitro* propagated R-1 and R-1,M cells with respect to their growth characteristics and their intrinsic radiosensitivity. For practical reasons, the present experiments were performed with different samples of cultured R-1,M cells collected from weekly *in vitro* passages, numbers 370 up to 473.

From aseptically isolated fragments of the mesentery of a germfree female WAG/Rij rat, cells were grown *in vitro* and after successful cloning, a permanent line of cells (designated the MER-1 cell line) was established. This cell line was regularly tested for spontaneous malignant transformation by injecting 10^5 to 10^6 MER-1 cells per inoculum s.c. or i.p. into both the WAG/Rij rat and the BALB/c.nu nude mouse, and was shown to be negative from the 94th to the 160th passage.

Cell culturing and the in vitro *assay.*- normal and tumour cells were rout grown in monolayers by employing star culture techniques. Medium was made Hanks' balanced salt solution, Eagle's mal essential medium and supplemented 20 volume % heat-inactivated calf serum (medium). Cells grown for experiments harvested by trypsinization from 3 to 5 cultures in glass culture flasks. The F cells of several flasks were pooled, wa resuspended in 25 ml of fresh HEC me and the number of cells present in this suspension counted. Similarly, stock su sions of MER-1 cells were prepared i quired. If R-1,M tumour cells were t used for *in vivo* measurements of radi dose response relationships, 20 ml of stock suspension was transferred to a p culture bottle and irradiated before fu dilution. Subsequently, for each typ experiment, series of suspensions with gr concentrations of tumour cells were prep by stepwise dilution with HEC mediur certain experiments, each of these di suspensions was admixed with normal M cells or F(R-1,M) "feeder" cells to a concentration of 10^6 admixed cells/ml.

At the start of each experiment, the tion of clonogenic cells present in the unirr ted stock cultures was evaluated in Fa plastic tissue culture flasks. These fixed after 8 days and colonies conta more than 50 cells were counted. Simil the fractions of surviving clonogenic cel the irradiated stock suspensions were ass *in vitro* for comparison with the *in vivo* a. To study the dose response relationshi *in vitro* cultured R-1,M cells, the cells irradiated with single doses of X-rays, 2 h after replicate plating of approp numbers of cells from stock suspensions.

The in vivo *assay.*—For TD_{50} as individual animals received two subc neous injections of 0·1 ml, one per flan one of the graded concentrations of R cell suspensions prepared as described al In a standard experiment, 6 different centrations of cell suspensions were prep and 6 groups of at least 5 animals per g were used in the assay. Only female WAG rats were used, but both sexes of BAL nu mice* were used, being randomly assi

* The nude mice were from CBA genetic stock and had been backcrossed with BALB/c for 4 genera They were produced under strict reverse barrier conditions in an SPF rodent colony and were ke conventional mouse quarters after the inoculation of the tumours (Van Bekkum *et al.*, 1978).

to different treatment groups. The animals used were aged 8 to 10 weeks. They were examined for the presence of tumours and, when positive, tumour volumes were measured once or twice weekly. The period of observation was usually 60 or 100 days, respectively, in BALB/c.nu mice or WAG/Rij rats.

TD_{50} values were calculated by means of probit analysis.

Irradiations.—Stock suspensions of R-1,M were irradiated with a 300 kV Philips-Müller X-ray generator, at 10 mA, dose rate 500 rad/min, HVL 2·0 mm Cu. For preparing feeder cells, a ^{137}Cs source was used to give 40 Gy. All suspensions were irradiated at room temperature.

RESULTS AND DISCUSSION

TD_{50} assays for unirradiated clonogenic R-1,M cells

A summary of results on tumour incidence for unirradiated R-1,M cells, assayed in the WAG/Rij rat and in the BALB/c.nu mouse, is given in Fig. 1. Curves 1 and 4 represent data obtained when only R-1,M clonogenic cells were injected, giving TD_{50} values (and their 95% confidence intervals) of 6945 (3586–13452) cells and 5267 (3362–8250) cells, in two species, *i.e.* not significantly different. Apparently the combined influence exerted by local conditions and general host factors is similar

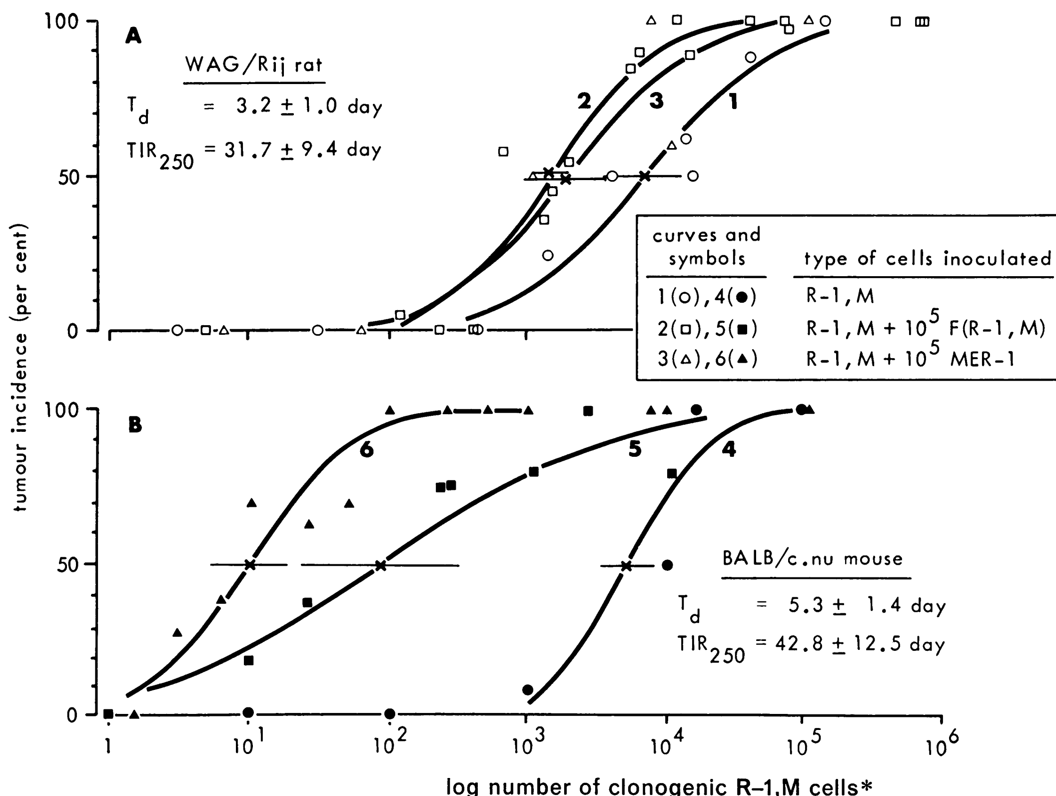

FIG. 1.—Relationships between the % tumour incidence and the log-number of inoculated clonogenic R-1,M cells assayed in the WAG/Rij rat (panel A) and the BALB/c.nu mouse (panel B). The symbols refer to the local conditions of the inoculated tumour cells which were altered by admixing heavily irradiated tumour cells, F(R-1,M) or phagocytic, normal MER-1 cells. TD_{50} values and their 95% confidence intervals are represented by × and horizontal bars, respectively. Inserts show values for tumour doubling time, Td, of tumours measuring 250 mm^3 and the time required for attaining this volume after transplantation of 10^5 tumour cells per inoculum. The growth characteristics are practically similar in the two hosts. *Assayed *in vitro*.

in the syngeneic and the allogeneic host. With respect to the conditions for macroscopic tumour growth, the properties of the two species also seem to be quite comparable. This is shown by the similar values for the doubling time, T_d, of tumours measuring 250 mm^3 and the time, TIR_{250}, required to reach that volume after inoculation of 10^5 R-1,M clonogenic cells (see Fig. 1, inserts of panels A and B).

Nevertheless, in both animals less than one cell in 10^3 leads to the growth of a tumour. For the WAG/Rij rat, immunization experiments performed by Tannock & Van Bekkum (1972) did not provide

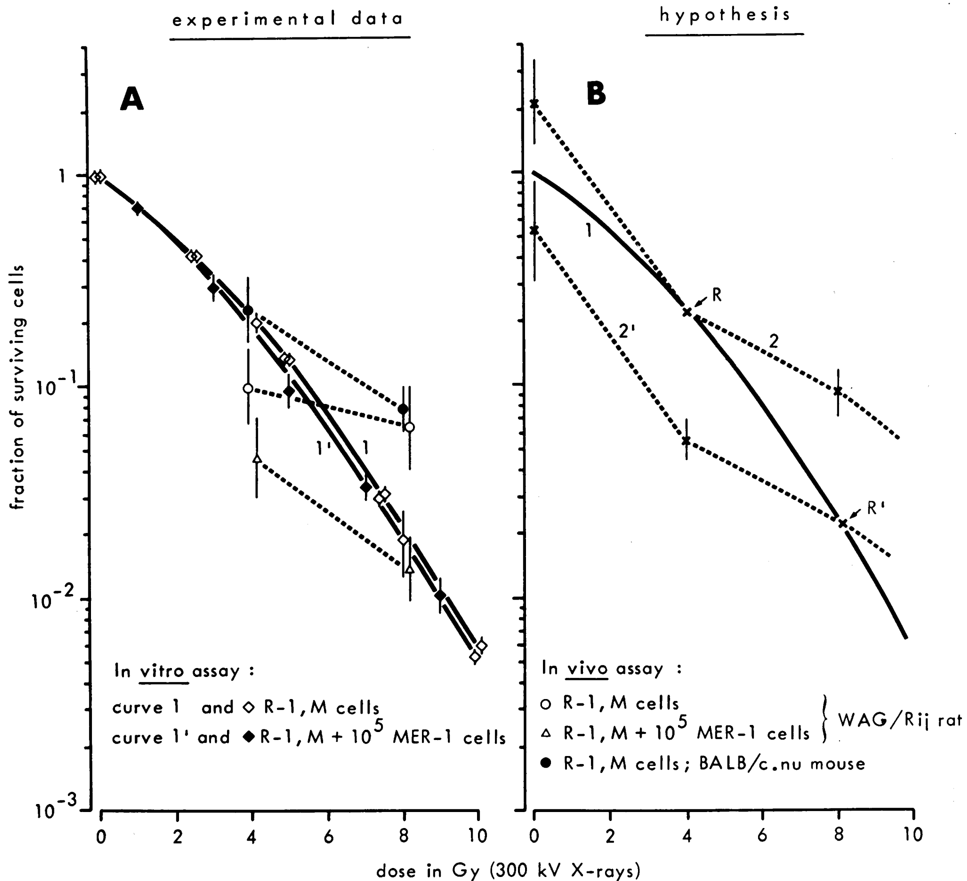

FIG. 2.—Comparison of dose response relationships for R-1,M tumour cells assayed *in vitro* and *in vivo*. Panel A.—Experimental data. *In vitro* assays performed with R-1,M cells grown in single culture (◇ and curve 1) and grown in mixed cultures (◆ and curve 1). The cells were plated *in vitro* and then irradiated with 300 kV X-rays. Experiments *in vivo* were performed by transplanting only R-1,M cells into the WAG/Rij rat (○) and the BALB/c.nu mouse (●), while R-1, M cells admixed with 10^5 MER-1 cells per inoculum were transplanted into the WAG/Rij rat (△). Vertical bars represent standard errors of the mean.

Panel B.—Comparison of experimental data obtained by the plating technique *in vitro* (curve 1) and by *in vivo* assays which have been recalculated to fit points R and R′ on curve 1, corresponding with fractions of cells surviving 4 and 8 Gy, respectively. Recalculation accords with the relationships $s_R = k \times s_X$ where k is a constant corresponding to values represented by R or R′; s_X is the fraction surviving cells measured by the *in vivo* assay and s_R the recalculated value.

Curves 2 and 2′ are the results of the recalculated experimental data fitting points R and R′, respectively. This method allows comparisons of the slopes of curves 1 and 2, or 2′, at points R and R′, respectively.

conclusive evidence as to whether humoral transplantation antigens influence the tumour incidence after inoculation of R-1,M cells in the syngeneic host.

The influence of F(R-1,M) "feeder" cells and viable phagocytic MER-1 cells, admixed with the clonogenic R-1,M cells, on the tumour incidence is demonstrated in Fig. 1. From the corresponding values for TD_{50}, *i.e.* 1428 (1091–1868) and 1893 (960–3732), respectively, it can be concluded that both types of admixed cells improve the local conditions for clonogenic expression of the tumour cells in the syngeneic rats. The TD_{50} is reduced by a factor of 3–4.

Significantly greater enhancement was observed when the same experiment was performed in the allogeneic BALB/c.nu mouse system (Fig. 1). Values for TD_{50} calculated from these data are 87 (23–331) and 10 (5–19), respectively. Unfortunately, the data obtained with the R-1,M feeder cells are not entirely reliable because of an outbreak of mouse hepatitis; therefore, it is not possible to conclude whether the MER-1 cells are more effective than the F(R-1,M) "feeder" cells in increasing the tumour incidence in this particular system. However, calculations show that viable MER-1 cells are capable of reducing the TD_{50} by a factor of 600 in the BALB/c.nu mouse.

The effects of the phagocytic MER-1 cells seem, in some respects, to contrast with those obtained from *in vitro* experiments which showed that the capacity for proliferation of R-1,M cells was reduced by the MER-1 cells when grown in mixed cultures. Therefore, a complementary hypothesis may be proposed, *i.e.* that, *in vivo*, the phagocytic MER-1 cells not only act against the tumour cells but also against the immune cells which otherwise would kill many more tumour cells.

TD_{50} assays with clonogenic cells after irradiation in vitro

In Fig. 2 dose response curves are shown for R-1,M cells irradiated *in vitro* after replicate plating. Curve 1 was constructed from data on numbers of colonies grown in cultures with medium enriched with F(R-1,M) "feeder" cells; curve 1′ was constructed from data in which R-1,M cells were cocultured with viable MER-1 cells (Hermens & Madhuizen, 1978). The data can be fitted by a common curve, within the error limits and, therefore, it may be concluded that the intrinsic radiosensitivity of R-1,M cells *in vitro* is not significantly influenced by the presence of MER-1 cells. The values for D_Q and D_0 derived from these curves are 2·5 and 1·3 Gy, respectively and are in good agreement with those reported by Barendsen & Broerse (1969).

From these dose response curves the surviving fractions after *in vitro* irradiation with single doses of 4 and 8 Gy of 300 kV X-rays are expected to be 0·22 and 0·022, respectively. In Fig. 2A these values are compared with those calculated from the TD_{50} data after irradiation *in vitro* but assayed in the WAG/Rij rat or the BALB/c.nu mouse. The results obtained with the *in vivo* assay are in striking variance with those obtained *in vitro*. Firstly, the fractions of surviving clonogenic cells differ significantly at one or both of the dose levels studied. In this respect, the results obtained with R-1,M cells assayed in the BALB/c.nu mouse are no better or worse than those obtained by assay in the WAG/Rij rat. Secondly, the slope of each of the dotted curves from all 3 *in vivo* assays (see Fig. 2A) is less steep than that from the *in vitro* assays. This difference in slopes is more clearly shown in Fig. 2B, as described in the legend.

It may be concluded from these observations that assays of R-1,M tumour cell survival *in vivo* may yield results which are different from those measured by the *in vitro* plating technique. Especially at doses smaller than 10 Gy it may be difficult to derive meaningful values for D_Q and D_0. Moreover, the present data show that *in vivo* the expression of the clonogenic capacity of surviving R-1,M

clonogenic cells is influenced significantly by the local conditions; *e.g.* the presence of phagocytic MER-1 cells. Experiments are in progress to study in greater depth the usefulness of employing the BALB/c.nu mouse for measuring dose response relationships of allogeneic cell types.

The financial support of the Koningin Wilhelmina Fund given to this project is gratefully acknowledged by the author. He also wishes to thank Mr H. T. Madhuizen for his expert help.

REFERENCES

BARENDSEN, G. W. & BROERSE, J. J. (1969) Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. 1. Effects of single exposures. *Eur. J. Cancer*, **5**, 373.

HERMENS, A. F. & BARENDSEN, G. W. (1969) Changes of cell proliferation characteristics in a rat rhabdomyosarcoma before and after X-irradiation. *Eur. J. Cancer*, **5**, 173.

HERMENS, A. F. & BARENDSEN, G. W. (1977) Effects of ionizing radiation on the growth kinetics of tumors. In *Growth Kinetics and Biochemical Regulation of Normal and Malignant Cells* (The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute 29th Annual Symposium on Fundamental Cancer Research, 1976). Eds B. Drewinko and R. M. Humphrey. Baltimore: Williams and Wilkins Co. p. 531.

HERMENS, A. F. & MADHUIZEN, H. T. (1978) Comparison of sensitivity to X-rays of normal MER-1 cells and syngeneic R-1,M rhabdomyosarcoma cells grown *in vitro* in single and mixed cultures. *Int. J. Radiat. Biol.*, **34**, 560.

HEWITT, H. B. & WILSON, C. (1959) A survival curve for mammalian leukaemia cells *in vivo*. *Br. J. Cancer*, **13**, 69.

PUCK, TH. T. & MARCUS, P. I. (1956) Action of X-rays on mammalian cells. *J. Expl. Med.*, **103**, 653.

REINHOLD, H. S. (1965) A cell dispersion technique for use in quantitative transplantation studies with solid tumours. *Eur. J. Cancer*, **1**, 67.

REINHOLD, H. S. (1974) The influence of radiation on blood vessels and circulation. *Curr. Topics Radiat. Res., Quart.*, **10**, 3.

TANNOCK, I. F. & VAN BEKKUM, D. W. (1972) Personal communication.

VAN BEKKUM, D. W., KNAAN, S. & ZURCHER, C. (1978) High capacity of human bronchial cancer to grow in allogeneic nude mice. In *Annual Report REPGO-TNO*. Rijswijk, The Netherlands. p. 157.