

CLONOGENIC POTENTIAL OF HUMAN TUMORS

A hypothesis

J. TEPPER

In 1956 PUCK & MARCUS first generated an in vitro cell survival curve of mammalian cells using the techniques for culturing individual mammalian cells that they had developed previously. This was followed, in 1959, by HEWITT & WILSON who performed the first in vivo log-kill survival curve using a dilution assay technique. Since that time an enormous amount of radiobiologic research has been performed aimed at defining the details of the shape and characteristics of the cell survival curves, in the hope of applying insight gleaned from these experiments to produce a better strategy for treating human tumors.

A number of mathematical models have resulted from the radiobiologic research. An example is the multi-target, single-hit model of the form $S/S_0 = 1 - (1 - e^{-D/D_0})^N$. In this equation, S_0 equals the number of viable cells in the initial tumor population, S is the number of cells remaining after a dose of irradiation D , and D_0 and N are parameters reflecting the slope and shoulder of the cell survival curve. This model has been used by SUIT et coll. (1965) to examine the concordance between the tumor control dose in 50 per cent of mammary carcinomas (TCD_{50}) with the mathematical model. Using narrow bands for D_0 (3 to 3.25 Gy) and for the extrapolation number N (1 to 6), and assuming 10^6 clonogenic cells per mm^3 , they found a strong correlation between the experimentally determined single dose TCD_{50} and that predicted by the model.

Unfortunately, a correlation has not been ob-

served for human tumors, which are generally treated with multiple fractions and low dose per fraction. Several explanations for this discrepancy are possible: (1) inadequate information available on the slope of the initial portion of the cell survival curve, (2) differences between the in vitro and in vivo cell survival curves, (3) absence of knowledge of the precise effects of fractionation, and (4) ignorance of the exact number of clonogenic cells in human tumors.

In order to explain the absence of correlation in human tumors, the dose control curves for human tumors treated in vivo with fractionated radiation therapy were analysed. From these results, the number of clonogenic cells in squamous cell carcinomas measuring 2 to 5 cm in diameter of the head and neck in humans was estimated. This analysis resulted in the hypothesis now proposed that there are a smaller number of clonogenic cells in human tumors than is usually assumed.

Material and Methods

Data have been pooled from numerous reports in the literature (SHUKOVSKY 1970, FLETCHER 1973, SHYKOVSKY & FLETCHER 1973, STEWART & JACKSON 1975, PEREZ et coll. 1976, SHUKOVSKY et coll. 1976, SPANOS et coll. 1976). The published information has been segregated for tumor size. For this analysis, medium-sized (2-5 cm in diameter)

Accepted for publication 26 February 1981.

squamous cell carcinomas of the head and neck region were included, for two reasons. First, these were considered to represent clinically a relatively homogeneous group of tumors, and secondly, good information on dose versus local control was available.

The criteria of local control varied somewhat by institution, but generally included no evidence of local disease for at least 2 years after local therapy. All doses were initially expressed in tumor ret [$NSD = \text{dose}/N^{0.24} \times T^{0.11}$] as defined by ELLIS (1969) with dose expressed in cGy. However, most of the institutions delivered the radiation at approximately 2 Gy per fraction, 5 fractions per week. The dose versus local control curves were then generated by a logit analysis and converted back to a Gy dose.

From these data, an attempt was then made to determine the number of viable clonogenic cells in the original tumor population. Initially the following assumptions were made: (1) the presence of one viable, clonogenic cell results in local tumor regrowth, this being an operational definition of a clonogenic cell, (2) each radiation fraction inactivates the same proportion of clonogenic cells, (3) the tumor populations from which the dose control curves were generated are homogeneous, and (4) the number of clonogenic cells remaining in a population of tumors at a given TCD can be determined by the Poisson distribution:

$$f(x) = \frac{\lambda^x e^{-\lambda}}{x!} \quad (1)$$

where $f(x)$ equals the proportion of tumors with x cells remaining at a certain dose; i.e. at the TCD_{50} , $f(0) = 1/2$ and at the TCD_{20} , $f(0) = 0.2$, etc., and λ = the mean number of cells remaining in the population of tumors at a certain dose. No assumptions were made regarding a specific mathematical model for the cell survival curve, and no estimates were made for parameters in the cell survival curve.

Results

The dose-local control data derived from the literature review, and the dose-local control curve obtained from the logit analysis of these data appear in Fig. 1. From this curve estimates of the TCD_{20} , TCD_{50} , and TCD_{80} were obtained, with

LOCAL CONTROL

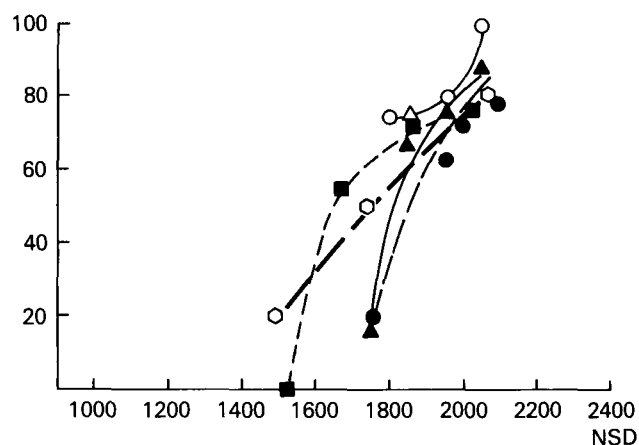


Fig. 1. Composite dose versus local control (per cent) curve for 2 to 5 cm diameter squamous cell carcinomas of the head and neck. T2 larynx, Christie (●). T2 tonsil, M. D. Anderson Hospital (○). T2 + T3 supraglottic, M. D. Anderson Hospital (▲). T2 + T3 base of tongue, M. D. Anderson Hospital (△). T2 + T3 tonsil, Washington University (■). Logit fit (○).

LOG (S/S₀)

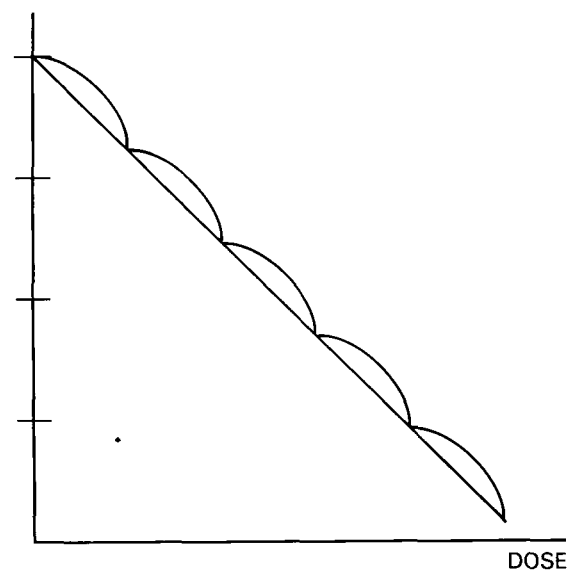


Fig. 2. Curve of log surviving cell fraction versus dose for multi-fraction radiation therapy. The cell survival curve for a single fraction can have an arbitrary shape, but the composite curve will be an exponential. $S/S_0 = e^{-D^1}$.

$TCD_{20} = 46$ Gy (1480 ret), $TCD_{50} = 58.5$ Gy (1740 ret), and $TCD_{80} = 75.5$ Gy (2047 ret). The doses for control outside these limits are too inaccurate for adequate analysis. Given assumption number 2 in Material and Methods, that each radiation fraction on average inactivates the same percentage of clonogenic cells in a tumor, the survival curve for

multi-fraction irradiations can be expressed in the form

$$S/S_0 = e^{-Dt} \quad (2)$$

where S_0 is the initial number of clonogenic cells in the tumor, S is the number of clonogenic cells remaining after a total dose of radiation D given in a multi-fraction course, and t is the slope of the composite survival curve (cf. Fig. 2). This equation is accurate regardless of the shape of the individual cell survival curve.

Then, at the $TCD_{20}=46$ Gy, from eq. (1)

$$f(0) = \frac{\lambda_1^0 e^{-\lambda_1}}{0!} = 0.2 \quad \text{thus} \quad \lambda_1 = 1.61$$

and likewise at the $TCD_{80}=75.5$ Gy

$$f(0) = \frac{\lambda_2^0 e^{-\lambda_2}}{0!} = 0.8 \quad \text{thus} \quad \lambda_2 = 0.223$$

At the TCD_{20} , from eq. (2), $S/S_0 = e^{-46t}$, and since $S = \lambda$ the mean number of clonogenic cells remaining, $S = \lambda = 1.61$.

$$\text{Therefore} \quad \frac{1.61}{S_0} = e^{-46t} \quad (3)$$

likewise, at the TCD_{80} $S/S_0 = e^{-75.5t}$

$$\text{and} \quad S = \lambda_2 = 0.223$$

$$\text{thus} \quad \frac{0.223}{S_0} = e^{-75.5t} \quad (4)$$

Combining eqs (3) and (4)

$$\frac{1.61}{0.223} = \frac{e^{-46t}}{e^{-75.5t}}$$

and solving for t , $t = 6.70 \times 10^{-2}$

Substituting t into eq. (3), $S_0 = 35$.

Thus, this calculation produces an estimate of 35 clonogenic cells in a medium-sized tumor, a much lower estimate than is generally made.

One of the initial 4 assumptions, that all the tumors from which the local control curve was derived are homogeneous in terms of cell number and sensitivity, is certainly not entirely true. In order to evaluate the effect of a large variation in the number of clonogenic cells in the tumor population from which the dose-local control curve

was obtained, it may be imagined that the curve is composed of 2 populations of tumors, where the number of clonogenic cells in one population (group 1) is 20 times that in the second population (group 2), or $S_{01}=20 S_{02}$. This should be a good estimate for the variability of cell number, since a variation in tumor diameter from 2 to 5 cm should produce a volume variation of a factor of 16. As a good approximation, group 2 should have a 40 per cent local control at the TCD_{20} for the whole population, and group 1 zero per cent control; at the TCD_{80} for the entire tumor population group 1 should have approximately 60 per cent local control and group 2 approximately 100 per cent control.

It is then possible to substitute

$$f(0) = 0.4 = e^{-\lambda_2} \quad \text{thus} \quad \lambda_2 = 0.916$$

$$\text{and} \quad f(0) = 0.6 = e^{-\lambda_1} \quad \text{thus} \quad \lambda_1 = 0.511$$

$$\text{and since} \quad S_{01} = 20 S_{02} \quad \frac{0.916}{2.56 \times 10^{-2}} = \frac{e^{-46t}}{e^{-75.5t}}$$

$$\text{then} \quad t = 1.21 \times 10^{-1}$$

$$\text{and} \quad S_{01} = 4.86 \times 10^3$$

$$\text{and} \quad S_{02} = 2.43 \times 10^2$$

Thus, although a large variation in cell number was assumed, the total number of clonogenic cells estimated in the individual tumors is still quite small.

It is more difficult to estimate the effect of a heterogeneous population of tumors with varying values of t (the slope in Fig. 2). Assuming two separate tumor populations with sensitivities of t_1 and t_2 , if $t_1 > 1.64 t_2$, no positive value of t would fit the data (a negative value for t would mean increased survival with increasing dose). If $1.55 t_2 < t_1 < 1.64 t_2$, then S_0 quickly approaches infinity. However, where $t_1 = 1.55 t_2$, then $S_0 = 1.84 \times 10^4$, still a small value of S_0 .

The values obtained from the logit fit of the clinical data could also be criticized, since they produce a more shallow curve than would be expected from a hand fit. The calculations could be repeated using much tighter bounds on the TCD_{20} and TCD_{80} using values of 55.5 Gy (1675 ret) and 71.5 Gy (1975 ret), respectively. Using this difference of 16 Gy between the TCD_{20} and TCD_{80} , S_0 is calculated as 1.53×10^3 cells, assuming no tu-

mor heterogeneity. The calculation of S_0 assuming a 20-fold variation in clonogenic cell number, results in $S_{0_1} = 4.48 \times 10^6$ and $S_{0_2} = 2.24 \times 10^5$.

A value of D_{37} (the dose required to reduce the surviving fraction to $1/e$ of its initial value) can also be estimated from the value of t calculated in eq. (2), since $D_{37} = 1/t$. Thus, for the situation where no heterogeneity of the tumor population is assumed, $t = 6.70 \times 10^{-2}$ and $D_{37} = 14.9$. If a 20-fold variation in the number of clonogenic cells is assumed, $t = 1.21 \times 10^{-1}$ and $D_{37} = 8.3$. Using the values derived from the hand-fit of the clinical data, the D_{37} is 4.5.

One way to estimate the reasonableness of these results is to analyse them in terms of the conventional cell survival curve $S/S_0 = 1 - (1 - e^{-D/D_0})^N$. If reasonable values for D_0 and N of 1.5 and 4, respectively, are estimated, the D_{37} for a single fraction of radiation is 3.3 Gy, which is somewhat lower than the D_{37} estimated for the hand-fit curve with 20-fold variation in cell number. However, this calculation assumes a D_0 which would be characteristic of a completely oxic cell population.

Discussion

One of the least satisfying aspects of the radiation biology research of the past twenty years is the inconsistency of the cell survival data and the control of human tumors. In humans, relatively low doses of radiation have been delivered over multiple fractions over several weeks compared with high dose, single fraction irradiation in most animal tumor experiments. Many authors have assumed that palpable tumors contained about 10^6 clonogenic cells per mm^3 (SUIT et coll. 1965, SHACKNEY et coll. 1978) which would be equivalent to more than 10^9 cells for tumors with average diameters of more than 1 cm. This is based on animal tumor experiments where a correlation has been found between theoretic calculations from cell survival curves and laboratory experiments and on estimates of the size of tumor cells. However, the doses needed to control even small animal tumors are far in excess of those needed to control comparably sized human tumors, implying a relatively smaller number of clonogenic cells in human tumors than in murine tumors.

It is not possible to explain the inactivation of such large numbers of cells at clinical dose levels from the multi-target, single-hit model or varia-

tions of this model and the experimentally determined values of D_0 and N . Even assuming a tumor with a $D_0 = 1.5$ and $N = 4$, one could only explain the inactivation of approximately 10^5 cells when treating at 2 Gy per day to a cumulative dose of 64 Gy.

In order to explain this discrepancy, some researchers have postulated models that would produce an increased slope in the initial portion of the cell-survival curve (BARENSEN 1962), as $S/S_0 = e^{-(\alpha D + \beta D^2)}$. This model assumes single-hit killing in the initial portion of the curve, and consequently predicts that a higher proportion of the cells will be killed at low dose fractions. None of these models, however, have explained satisfactorily the control of human tumors. Thus, it seemed worthwhile to examine data on the control of human tumors versus dose to see how reasonable the estimates of the number of clonogenic cells in human tumors have been. Even assuming a large variation (20-fold) in the number of clonogenic cells in the tumors forming the dose-local control curve, the largest estimate calculated for the number of cells in tumors with diameters of 2 to 5 cm is 5×10^6 .

The hypothesis presented can be criticized on the grounds that the mathematical results obtained can be explained by factors other than a small number of clonogenic cells. The calculated results follow directly from the fact that the dose-local control curve presented for medium-sized human squamous cell carcinomas has a shallow slope. Factors which may determine this slope are as follows: (1) the number of clonogenic cells initially present in the tumor and the statistical fluctuation in the dose needed to inactivate all of them (the hypothesis being presented, (2) variation among tumors in the initial number of clonogenic cells which must be inactivated, (3) variation in radiation sensitivity among the different tumors composing the dose-local control curve, (4) variation in the hypoxic fraction and in the kinetics of re-oxygenation, (5) change in the cellular radiation sensitivity between early and late radiation fractions, (6) variation in tumor proliferation during the course of treatment, (7) variations in the dose estimation and the treatment technique, and (8) variations in the immunogenicity of the tumors and in the immunologic potency of the host.

These factors cannot all be excluded as possible explanations for the results presented, but an attempt has been made to explain why they do not

negate the conclusions of the present report. Point 2 has been addressed, and it has been shown that even large (20-fold) variations in the number of clonogenic cells between tumors do not negate the hypothesis. Point 3 has also been analysed earlier in the article. Parameters of cell sensitivity can be defined which would result in a large number of clonogenic cells, but this variation would need to be defined tightly to produce the clinical data which would demonstrate a large number of clonogenic cells. This model allows cell sensitivity to vary (i.e., a variation in t) by a factor of 1.5 without affecting significantly the conclusions. In addition, *in vitro* experimental data by WEICHSELBAUM *et coll.* (1976) show only a small difference in inherent radiation sensitivity between tumor systems of different clinical radiation curability. Point 4 seems an unlikely explanation for the results, as a significant amount of experimental data shows that the proportion of hypoxic cells remains relatively constant during a course of fractionated radiation therapy (VAN PUTTEN & KALLMAN 1968). Also, the results are affected minimally if the proportion of hypoxic cells (or any other parameter of sensitivity to radiation) varies over the first few fractions but remains relatively constant during the latter part of the therapy. For this same reason, only a marked change in sensitivity between early and late fractions (point 5) would substantially affect these calculations. If the sensitivity (as expressed by t , the slope) increased in later fractions (as might occur with a smaller tumor mass and a smaller hypoxic fraction), the calculations mentioned would show an even larger number of clonogenic cells than are in fact present. Variations in tumor proliferation (point 6) can be accounted for in the same manner as variation in the number of clonogenic cells present in different tumors. This variation would have to cause the variation in clonogenic cell number to be greater than a factor of 100 to impact significantly on the results. Variations in estimation of dose and treatment technique (point 7) could be important if there were large errors in quoted doses. This should be minimal, as most of the clinical data are consistent with each other, and most come from one institution (M. D. Anderson Hospital) which has excellent physics support and a consistent treatment philosophy. Tumor immunogenicity (point 8) is unlikely to be important, as little evidence exists to support immunogenicity of

squamous cell carcinomas of the head and neck. In addition, all the variations discussed should be minimized by the attempt to analyse only tumors of similar size (2 to 5 cm in diameter or volume variation of a factor of 15), of similar type (squamous cell carcinomas) and from similar sites (head and neck). Indeed, the variations mentioned are necessary to produce results which do not show an unreasonably low number of clonogenic cells in these tumors.

The idea that there may be relatively few clonogenic cells in human tumors is not new and has been postulated previously by BUSH & HILL (1975). Their hypothesis, however, is not based on multi-fraction irradiation of human tumors. They do point out that the number of clonogenic cells does not necessarily correspond to the number of potentially clonogenic cells present in the initial tumor. There may be a large number of identical cells, each of which has a probability of less than 1.0 of producing a viable tumor. The transformation from a cell to a palpable tumor could be influenced by many factors, including the vascularity of the tumor bed, the presence of appropriate nutrients, the immunologic status of the host, cell loss factor (STEEL 1967) and the intrinsic characteristics of the tumor itself. Regardless of which, if any, of these factors may be important, the fact that the cell population needs to be decreased only by a factor of 10^6 in order to sterilize a tumor locally could have important radiation therapeutic and chemotherapeutic ramifications.

These calculations are also consistent with other information in the literature. It has long been reported that many tumor cells circulate in patients with breast carcinoma and other tumors (SALSBURY 1975). The fact that these circulating cells often do not produce metastases is evidence that they are not all clonogenic in the functional sense (SALSBURY). There are data in the literature on the control of human skin carcinoma (HALE & HOLMES 1947) and Hodgkin's disease (KAPLAN 1966) which, if calculated as mentioned, show an even smaller clonogenic population than was calculated for the epidermoid carcinoma of the head and neck. Indeed, it could be speculated that the major reason why chemotherapy for Hodgkin's disease is effective is the relatively small number of clonogenic cells both calculated and microscopically identified. It is also possible that the low plating efficiency observed by workers as

SALMON et coll. (1978) in culturing human tumor cells in vitro is not necessarily a function of the plating technique, but rather of the limited clonogenic potential of the cells being plated.

Although the data presented certainly cannot prove that a relatively small number of clonogenic cells exists in human tumors, they do present evidence to support that hypothesis. If this hypothesis is true, it may affect some of the basic treatment strategies which are formulated and clinically used in the treatment of malignancies with chemotherapy or radiation therapy.

SUMMARY

Conventional radiation therapy of medium-sized (2 to 5 cm diameter) squamous cell carcinomas of the head and neck has resulted in data which were pooled to produce a curve of local control versus dose. From this curve, estimates were made of the number of clonogenic cells in these tumors. These calculations demonstrate a much smaller number of clonogenic cells than is usually assumed for human tumors. It is postulated that a relatively small proportion of the cells in human tumors are clonogenic. The number of cells which need to be destroyed by radiation therapy or chemotherapy in order to produce tumor control may be much less than usually estimated.

ACKNOWLEDGEMENT

The author would like to thank many people who provided useful discussion and constructive criticism including Dr Eli Glatstein, Dr Elizabeth Travis, Dr Herman Suit and especially Dr Michael Goitein.

Request for reprints: Dr Joel E. Tepper, Department of Radiation Medicine, Massachusetts General Hospital, Boston, Massachusetts 02114, USA.

REFERENCES

- BARENDSSEN G. W.: Dose survival curves of human cells in tissue culture irradiated with alpha-, beta-, 24-kV x and 200-kV x-radiation. *Nature* 193 (1962), 1153.
- BUSH R. S. and HILL R. J.: Biologic discussions augmenting radiation effects and model systems. *Laryngoscope* 85 (1975), 1119.
- ELLIS F.: Dose, time and fractionation. A clinical hypothesis. *Clin. Radiol.* 20 (1969), 1.
- FLETCHER G. H.: Clinical dose response curves of human malignant epithelial tumors. *Brit. J. Radiol.* 46 (1973), 1.
- HALE C. H. and HOLMES G. W.: Carcinoma of skin. Influence of dosage on the success of treatment. *Radiology* 48 (1947), 563.
- HEWITT H. B. and WILSON C. W.: A survival curve for mammalian cells irradiated in vivo. *Nature (London)* 183 (1959), 1060.
- KAPLAN H. S.: Evidence for a tumoricidal dose level in the radiotherapy of Hodgkin's disease. *Cancer Res.* 26 (1966), 1221.
- PEREZ C. A., LEE F. A., ACKERMAN L. V., KORBA A., PURDY J. and POWERS W. E.: Carcinoma of the tonsillar fossa. Significance of dose of irradiation and volume treated in the control of the primary tumor and metastatic neck nodes. *Int. J. Radiat. Oncol. Biol. Phys.* 1 (1976), 817.
- PUCK T. T. and MARCUS P. I.: Action of x-rays on mammalian cells. *J. exp. Med.* 103 (1956), 653.
- SALMON S. E., HAMBURGER A. W., SOEHNLEN B., DURIE B. G. M., ALBERTS D. S. and MOON T. E.: Quantitation of differential sensitivity of human-tumor stem cells to anticancer drugs. *New Engl. J. Med.* 298 (1978), 1321.
- SALSBURY A. J.: The significance of the circulating cancer cell. *Cancer Treat. Rev.* 2 (1975), 55.
- SHACKNEY S. E., MCCORMACK M. D. and CUCHUEAL G. J.: Growth rate patterns of solid tumors and their relation to responsiveness to therapy. An analytical review. *Ann. intern. Med.* 89 (1978), 107.
- SHUKOVSKY L. J.: Dose, time, volume relationships in squamous cell carcinoma of the supra glottic larynx. *Amer. J. Roentgenol.* 108 (1970), 27.
- and FLETCHER G. H.: Time dose and tumor volume relationships in the irradiation of squamous cell carcinoma of the tonsillar fossa. *Radiology* 107 (1973), 621.
- BAEZA M. R. and FLETCHER G. H.: Results of irradiation in squamous cell carcinomas of the glosso-palatine sulcus. *Radiology* 120 (1976), 405.
- SPANOS W. J. JR, SHUKOVSKY L. J. and FLETCHER G. H.: Time, dose and tumor volume relationships in irradiation of squamous cell carcinomas of the base of the tongue. *Cancer* 37 (1976), 2591.
- STEEL G. G.: Cell loss as a factor in the growth rate of human tumors. *Europ. J. Cancer* 3 (1967), 381.
- STEWART J. G. and JACKSON A. W.: The steepness of the dose response curve both for tumor cure and normal tissue injury. *Laryngoscope* 85 (1975), 1107.
- SUIT H. D., SHALEK R. J. and WETTE D.: Radiation response of C₃H mouse mammary carcinoma evaluated in terms of cellular radiation sensitivity. *In: Cellular radiation biology*, p. 514. Williams and Wilkins, Baltimore 1965.
- VAN PUTTEN L. M. and KALLMAN R. F.: Oxygenation status of a transplantable tumor during fractionated radiotherapy. *J. nat. Cancer Inst.* 40 (1968), 441.
- WEICHELBAUM R. R., EPSTEIN J., LITTLE J. B. and KORNBLITH P. L.: Inherent cellular radio sensitivity of human tumors of varying clinical curability. *Amer. J. Roentgenol.* 127 (1976), 1027.