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EXPERIMENTAL CHEMOTHERAPY STUDIES: INTERCOMPARISON OF ASSAYS

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Summary.—In a limited number of experimental tumour systems the response to chemotherapy has been measured in terms of both cell survival and tumour growth delay. Both of these endpoints have complicating factors which lead to problems in interpretation of results. The time after drug administration at which cell survival is measured can be of predominant importance. If the time is too short, drug action may be incomplete and recovery from potentially lethal damage may still be occurring. If the time is too long, proliferation of surviving clonogenic cells may have begun. Tumour growth delay on the other hand is likely to be influenced by the effect of the chemotherapy on the host, as well as on the tumour. This may be particularly important when the tumour is significantly immunogenic. The rate of regenerative proliferation of the surviving clonogenic cells can be different following treatment with different cytotoxic drugs, hence resulting in different periods of growth delay from the same initial level of cell killing. Also some agents appear to be significantly cytostatic, producing considerable growth delay in the absence of cell killing. The available data from the literature relevant to these points are reviewed. New data are also presented for the RIF-1 tumour and for multicellular tumour spheroids of the EMT6/Ca/VJAC line.

THERE ARE 3 main differences between ionizing radiation and cytotoxic drugs relevant to the question of comparison of response endpoints. Firstly, the period of drug action is not precisely defined in time as is exposure to external beam irradiation. Drugs vary in their stability and rate of excretion and metabolism so that the period of time following drug administration over which damage is inflicted upon target tissues is not well known. Secondly, whereas experiments designed to examine tumour response to radiation usually employ a beam collimated to expose only the tumour and immediately adjacent tissues, the response to drugs nearly always employs systemic administration and whole body exposure. The possible systemic modification of the host by the drug is likely therefore to be a factor in tumour response. Thirdly, there is a wide variety of modes of action amongst the various cytotoxic drugs, and although some of these are in some ways analagous to that of ionizing radiation, others are very different. There is, therefore, no reason to expect that the time course of such events as damage fixation or repair will be the same for drugs and radiation.

For cytotoxic drugs, there are very few instances in experimental systems where established solid tumours can be cured by a drug alone in a single dose. This is because of the toxic effect upon the host animal of the doses which would perhaps achieve tumour control if they could be administered. The TCD_{50} endpoint is therefore not generally relevant to cytotoxic drug experiments. On the other hand, use of the generalized endpoint of "increase in lifespan", although clinically relevant, is likely to be misleading when used for solid experimental tumours unless the cause of death is determined in each animal. Spread of metastatic disease which may have begun before drug administration, and the effect of the drug on the pattern of metastasis may very well be involved in determining the lifespan of

TABLE I.—*A comparison of growth delay and clonogenic cell survival in 2 tumour systems following chemotherapy*

Tumour	Treatment	Growth delay	S. fraction at 24 h	Author
Lewis lung carcinoma	CCNU	~0	0·006	Stephens & Peacock (1977)
H-4-II-E rat hepatoma	Adriamycin 10 mg/kg	11 days	~1·0	Rowley *et al.* (unpublished)

TABLE II.—*Factors affecting relativity between cell survival and tumour growth delay*

Factors which will tend to make the treatment appear **more** effective if assayed by cell survival than by tumour growth delay	Factors which will tend to make the treatment appear **less** effective if assayed by cell survival than by tumour growth delay	Category
Assay carried out before recovery from potentially lethal damage is complete	Assay carried out before drug action is complete Assay delayed until after a preferential loss of killed tumour cells has occurred Assay delayed until after proliferation of surviving cells has commenced	"Time of clonogenic assay" effects
Toxicity of disaggregation procedure (either alone or additive with drug damage)	Colony formation by resistant "non-tumour" cells Artificial rescue of doomed cells	Artefacts of clonogenic assay procedures
Non-representative sample ← →	Non-representative sample	
Depression of immune response controlling tumour growth rate	Damage to "tumour bed"	Host effects
Depression of non-specific host resistance	General debilitation of host (weight loss?)	
Infiltration of tumour volume by host cells		
More rapid proliferation of clonogenic cells in treated than in control tumours	Less rapid proliferation of clonogenic cells in treated than in control tumours Induced cell-cycle delay	Kinetic factors involving surviving cells

the animal in a way which gives no indication of the effectiveness of the therapy against the primary tumour. The endpoints upon which I am, therefore, going to concentrate are the growth delay of the intact solid tumour and the surviving fraction of clonogenic cells. In Table I, I have brought together 2 recent sets of data showing how disparate the measured tumour response to chemotherapy can be if different endpoints are compared. I will attempt to explain in this paper how such enormous apparent discrepancies can arise.

In Table II the factors are summarized which are liable to affect the relationship between the assessment of treatment effectiveness using different endpoints. Several of these factors are concerned with the "time of assay" for clonogenic cell survival, *i.e.* the time between administration of the drug and the excision of the tumour in order to prepare a cell suspension. As I reviewed this particular point only a year ago (Twentyman, 1979) and my views on it remain unchanged, I will only summarize the conclusions of that review. Firstly, the time between drug treatment and assay must be sufficiently long that the cytotoxic action is complete; for some agents with long biological half lives, this can mean waiting 24 h or more. Secondly, if we are interested in the overall effect of therapy, then we must avoid modification of the measured cell survival by interfering with the recovery processes which occur in the undisturbed tumour (*i.e.*

"recovery from potentially lethal damage"). For some agents, it appears that such recovery occurs for at least 48 h after administration. If, however, consideration of these 2 factors leads to a prolonged delay in assay, then cell loss and regenerative proliferation will have occurred, and the population being assayed will not be equivalent to that present at the time of treatment. This will be a particular problem for agents such as vincristine which can cause considerable *in situ* lysis of cells within the tumour during the first 24 h after administration (Stephens & Peacock, 1978). In some circumstances, therefore, a single "correct" value for the surviving fraction of clonogenic cells present in the tumour at the time of drug administration cannot be determined. As a general rule, however, an assay of cell survival carried out at 24–48 h after drug administration is likely to give the best answer, provided that changes in cell yield are taken into account in addition to changes in clonogenic fraction.

In addition to these "time of assay" factors which need to be considered, there are a number of possible "artefacts" involved in the measurement of clonogenic cell survival which could clearly influence the result obtained. First of all, the yield of cells from the solid tumour into a single cell suspension, by whatever disaggregation procedure is used, is rarely 100%. For some tumours, yields of 25–50% may be obtained (Stephens & Peacock, 1978) whereas for others, the yield may be as low as 1% (Twentyman, 1977*b*). If the sample of cells obtained is not representative of the tumour cell population as a whole, then it is clear that the possibility of biased estimates of surviving fraction exists. A second problem arises from the fact that it is usually difficult when carrying out a haemocytometer count to distinguish with certainty between tumour cells and non-tumour cells present in the suspension. If one effect of a given treatment has been to alter this ratio at the time of preparation of the suspension, then a wrong estimate of surviving fraction is possible. It should be noted, however, that this particular problem will not apply if the results are expressed as "total clonogenic cells per tumour" after applying the appropriate corrections. Many enzymatic processes of tissue disruption involve severe damage to membranes, and toxicity by the actual disaggregation procedure may also occur, possibly to a greater extent in cells already damaged by cytotoxic drug. This is one possible explanation of the data reported by Rasey & Nelson (1980) showing different survival curves for EMT6 cells from tumours treated with cyclophosphamide or bleomycin and then disaggregated using 2 different procedures. In some circumstances, care must be taken to ensure that colonies produced *in vitro* from suspensions prepared from solid tumours do in fact arise from tumour cells. It has been shown that for the Lewis lung carcinoma, a proportion of the colonies formed in soft agar arise from macrophages, and furthermore, that the proportion of these colonies can be very different after treatment of the tumour (Stephens *et al.*, 1978). Finally, it has been postulated that cells which, in the tumour, would have died due to their distance from blood vessels may be artificially rescued by disruption of the tumour and exposure of all cells to optimal growth conditions. I should like, however, to emphasize that most of these factors are hypothetical and essentially no information exists to establish whether or not they are important in specific systems.

Referring back now to Table II, it is clear that there are a number of factors involving systemic effects of drug upon the tumour host which may modify the period of tumour growth delay following treatment. These have been extensively reviewed by Brown (1979) and will only be mentioned here. Both specific immune response and non-specific host resistance may be modified by drug treatment, and these factors may be involved in the regulation of growth rate, especially for significantly immunogenic tumours. Infiltra-

tion of the tumour volume by host cells following treatment can also occur rapidly in some tumour systems (Stephens *et al.*, 1978). A general debilitation of the host condition following high dose chemotherapy (often manifest in loss of body weight) may also impair tumour growth.

Having considered all these factors, which in one sense are artefacts of the experimental models used but which are none the less important in making a realistic comparison of endpoints, we are left with perhaps the central question of understanding how tumours respond to therapy: *i.e.* "Is the overall effect of therapy on the intact tumour a reflection of the effectiveness in killing the clonogenic tumour cells?" To answer this question, there are probably 2 aspects to consider. Firstly, can cytotoxic drugs also be cytostatic, to the extent that significant growth delay can be produced in a tumour? Secondly, how does the proliferation rate of the surviving clonogenic cells vary with the particular agent and with the degree of depletion of their numbers?

In order to approach these problems, I should now like to turn to the available data from the literature. I shall only use data obtained where growth delay and survival have been measured in the same series of experiments in the same laboratory. This restriction means that the amount of information is unfortunately very small. It has been necessary for me to decide how to plot the data relating the 2 endpoints and I have taken the straightforward way of plotting delay in time for mean tumour volume to reach a given multiple of the treatment volume (usually 4×) against the logarithm of the surviving fraction measured at or about 24 h after drug administration. Using this type of plot, it is then possible to deduce a mean doubling time for the surviving clonogenic cells:

$$\mathrm{Td} = \frac{\log 2 \times \text{growth delay}}{-\log \text{surviving fraction}}$$

This formula assumes that proliferation of surviving cells begins immediately after treatment, *i.e.* there is no drug-induced proliferation delay, and also that the post-treatment proliferation rate continues until the regrowing tumour reaches the treatment volume at which time it reverts to a pattern similar to the untreated controls at that volume.

Mentioning first our own work using a version of the EMT6 tumour, we found that for bleomycin, essentially no deviation of the growth curve was found for doses which gave a measured surviving fraction of 10^{-3} at 30 min after treatment (Twentyman, 1977*b*). We were subsequently able to show that this result was artefactual and that the cells were killed by being subjected to the process of disaggregation while bleomycin remained in the tumour. This, therefore, is one par-

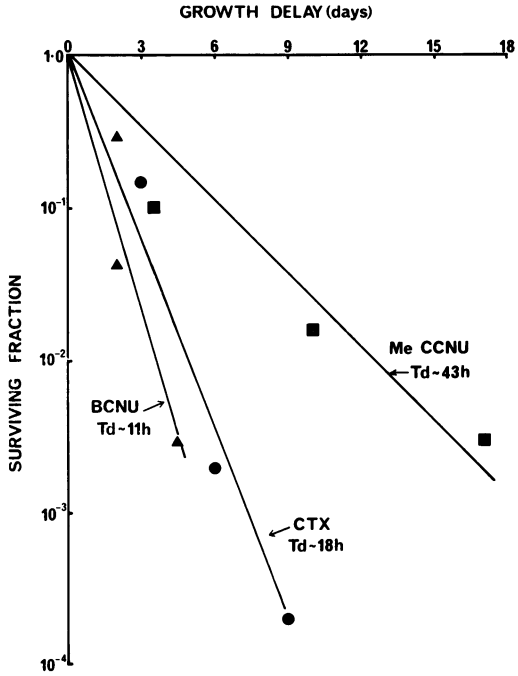

FIG. 1.—Growth delay *vs* surviving fraction for EMT6/Ca/VJAC tumours. Surviving fraction assayed at 24 h after drug administration. Redrawn from Twentyman (1977*a*, 1978). ▲, BCNU; ●, cyclosphosphamide; ■, MeCCNU. The lines drawn by eye are only an approximate fit to certain of the data points (see text).

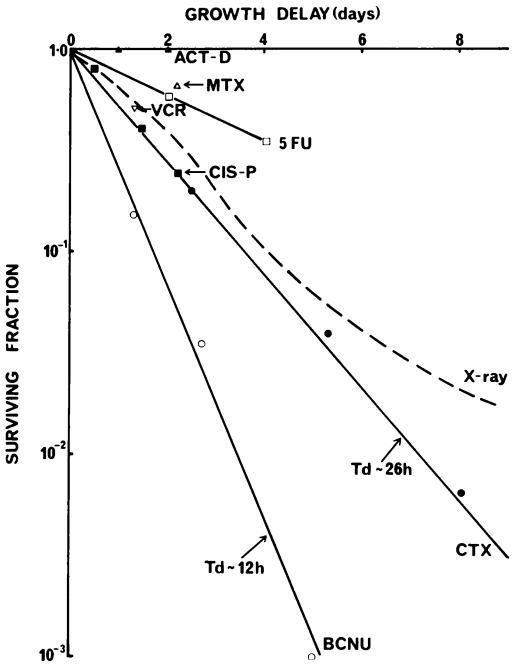

FIG. 2.—Growth delay *vs* surviving fraction for EMT6/SF tumours. Surviving fraction assayed at 24 h after drug administration. Redrawn from Begg *et al.* (1980). ○, BCNU; ●, cyclophosphamide; ■, cis-platinum; ▽, vincristine; □, 5-fluorouracil; △, methotrexate; ▲, actinomycin D. The points shown are values read off from lines drawn by the original authors to fit their data. The solid lines have been drawn by eye to fit the data for BCNU, CTX and 5FU. The broken line is drawn to fit a set of values read off as above.

ticular example of how "time of assay" can be of overriding importance.

No such artefact was found, however, for BCNU or cyclophosphamide (CTX), where recovery from potentially lethal damage also appeared to occur after treatment, changing the measured survival by a factor of 100× between 2 and 48 h after treatment. Again, therefore, "time of assay" is very important, but for these drugs, not in a way which has been shown to be artefactual (Twentyman, 1977*a*, 1978). The results comparing growth delay and surviving fraction measured at 24 h after treatment with BCNU, CTX or methyl CCNU are shown in Fig. 1.

It may be seen that the implied doubling times are about 11, 18 and 43 h at the higher doses of BCNU, CTX and MeCCNU respectively. A much wider range of drugs has recently been studied in a different sub-line of the EMT6 tumour by Begg and his co-workers (1980) (Fig. 2). As in my own study, BCNU again gives a very short implied doubling time of 12 h. For CTX and *cis*-dichlorodiamine platinum the value is around 26 h and for 5-fluorouracil the time is 60–70 h. Actinomycin D gave 2 days of growth delay for no apparent cell kill. This latter piece of information is particularly interesting in view of some recent unpublished data kindly supplied to me by Dr Roy Rowley for adriamycin, another intercalating agent, summarized in Table I. Using the H-4-II-E rat tumour system they observed that a single dose of 10mg/kg adriamycin produced a very considerable slowing of tumour growth followed eventually by a resumption of growth similar to that of untreated tumours. The growth delay was about 11 days. At no time after treatment, however, was any significant change in the surviving fraction of extracted cells seen. These observations appear likely to be a reflection of considerable proliferation delay induced by the drug. Recent studies by Dethlefsen *et al.* (1979) using flow cytometry have shown that following adriamycin treatment of a fast-growing C3H mouse mammary tumour, there is a pronounced and long-lasting decrease in the number of cells in DNA synthesis and in the mitotic index. There was, however, no increase in the number of "degenerative" cells. The authors concluded that the long delay seen in volumetric growth appears to be due to an extended cell-cycle rather than to extensive cell killing.

We have recently in our laboratory been carrying out a comparison of growth delay and measured surviving fraction in EMT6/Ca/VJAC tumour spheroids following chemotherapy *in vitro*. The methodology was similar to that described by Yuhas *et al.* (1977) and the treatment time was 1 h at 37°C. These data are

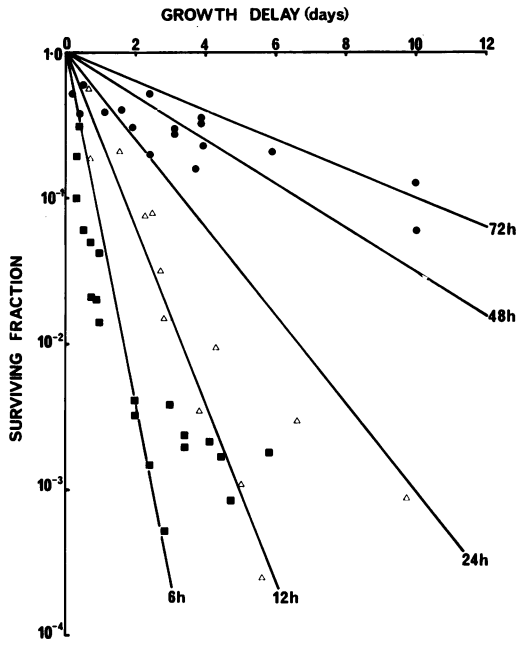

FIG. 3.—Growth delay *vs* surviving fraction for multicellular tumour spheroids (250–300 μm diameter) of the EMT6/Ca/VJAC line. Surviving fraction assayed at the end of 1h drug exposure. ●, adriamycin; △, nitrogen mustard (HN2); ■, BCNU. Each point represents the result of a single experiment, using 12 spheroids/group for growth delay determinations. The lines show where points would lie on the graph in order to correspond to the implied doubling times indicated.

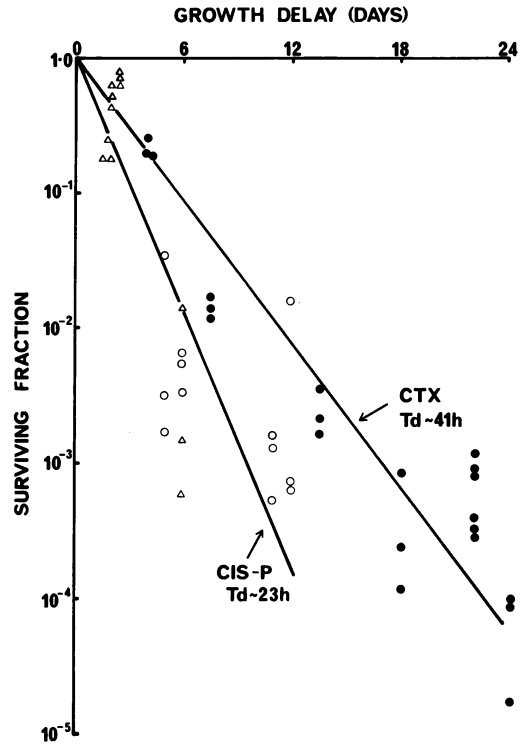

FIG. 4.—Growth delay *vs* surviving fraction for RIF-1 tumours. Surviving fraction assayed at 24 h after drug administration. △, BCNU; ○, *cis*-platinum; ●, cyclophosphamide. Each point represents the surviving fraction measured for a single treated tumour, plotted against the mean growth delay for a group of 6–8 similarly treated tumours in the same experiment. The lines are drawn by eye to fit the data for cis-platinum and cyclophosphamide.

for disaggregation of the spheroids immediately after drug exposure and it may be seen that the apparent doubling times cover a very wide range (Fig. 3). For BCNU, most points for lower drug doses imply doubling times of less than 6 h whereas for adriamycin, implied doubling times of 48–72 h are seen at higher drug doses. More recent data show that there is considerable apparent recovery from potentially lethal damage following BCNU and nitrogen mustard and that a delay in spheroid disaggregation leads to an increase in measured survival by at least a factor of 10× at higher doses. No change in measured survival with time occurs for adriamycin, however, and our spheroid studies therefore confirm the *in vivo* observations of relatively long growth delay for modest cell killing with adriamycin in the absence of any possible "host" effects.

Some data for a new *in vivo/in vitro* tumour system (RIF-I) are shown in Fig. 4. This is a radiation induced sarcoma of the C3H mouse which shrinks rapidly following treatment with radiation and some cytotoxic drugs (Twentyman *et al.*, 1980; Brown *et al.*, 1980). The Figure shows a comparison of growth delay *vs* surviving fraction measured at 24 h following CTX, BCNU or *cis*-p. This tumour is unresponsive by either end-

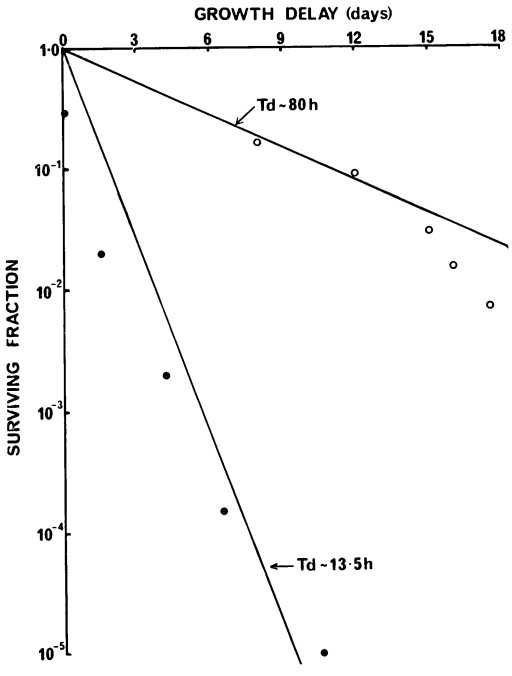

FIG. 5.—Growth delay *vs* surviving fraction for the LMC_1 rat tumour (open circles) and the Lewis lung tumour (closed circles) treated with cyclophosphamide. Surviving fraction assayed at 24 and 16 h respectively after drug administration. Redrawn from Moore & Dixon (1978) (LMC_1 tumour) and from Steel & Adams (1975) (Lewis lung tumour). The points shown have been read off from lines drawn by the original authors to fit their data. The upper solid line has been drawn to pass between the first 2 data points for the LMC_1 tumour. The solid lower line has been drawn to pass between the final 2 data points for the Lewis lung tumour (see text).

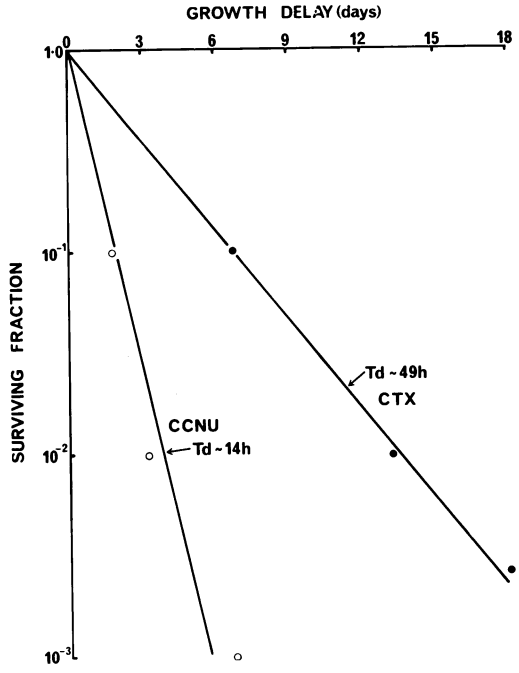

FIG. 6.—Growth delay *vs* surviving fraction for B16 melanoma. Surviving fraction determined at 18 h after drug administration (redrawn from Stephens & Peacock, 1977). ○, CCNU; ●, cyclophosphamide. The points shown have been read off from lines drawn by the original authors to fit their data. The lines have been drawn by eye to fit the points.

point to doses of BCNU which approach the maximum tolerated dose (except apparently in one experiment where, for unknown reasons, a relatively large response was seen). Considerable growth delay is obtained following treatment with the other 2 drugs, however, and the implied doubling time after CTX is almost twice that after *cis*-platinum.

Two more sets of data, both for CTX, are shown in Fig. 5. The data of Steel & Adams (1975) for the Lewis lung tumour, show an initial rapid fall in surviving fraction to 0·02 for a growth delay of 1·5 days (implied Td = 6·4 h), and then a more shallow fall (implied Td for the last 2 points considered alone of around 13·5 h). The LMC_1 rat tumour as used by Moore & Dixon (1978), however, shows the opposite pattern, with an initial shallow slope (implied Td around 80 h) giving way to a steeper slope. The volume doubling time of untreated tumours was about 2 days for the Lewis lung and 4 days for the LMC_1.

Finally, I want to turn to the data of Stephens & Peacock (1977) for the response of the B16 melanoma to CTX and CCNU. Here the implied doubling times are 49 h and 14 h respectively using the method of analysis which I have employed; the data are shown in Fig. 6. Stephens & Peacock, however, use a different method of analysis which gives values of 59 and 29 h respectively. This difference in the actual values does not

affect the general conclusion, confirmed by very careful time course studies of clonogenic cells *per tumour*, that the doubling time of clonogenic cells during tumour regeneration is very different for the 2 drugs.

The basis of the difference in method of calculating doubling times between myself and Stephens & Peacock (1977) is extremely important in considering whether the very short implied doubling times can possibly be correct. I assume that the proliferation rate of the clonogenic cells returns to normal when the regrowing tumours pass their initial treatment volume. Stephens & Peacock, on the other hand, assume that the doubling time of the clonogenic cells remains constant from immediately post-treatment until the regrowing tumour has reached 4× treatment volume. Their method certainly allows for short growth delays to be reasonably associated with relatively large depressions in surviving fraction, and there is evidence from Stephens & Peacock's (1977) data that this method may be correct for the B16 melanoma. Examination of the data of Hermens & Barendsen (1969) for the R1 rhabdomyosarcoma following radiation treatment, however, shows that in this system, a slowing down of the clonogenic cell regeneration occurs even *before* the tumour has regrown to the treatment volume. This is, therefore, a factor which is likely to vary from tumour to tumour and to greatly complicate the analysis of endpoint comparison. The importance may be demonstrated by consideration of the data given by Stephens & Peacock (1977) for the Lewis lung tumour following treatment with CCNU, and shown in Table I, *i.e.* essentially no growth delay for a measured survival of 0·006. Let us assume that the doubling time of the control tumours is about 2·5 days during growth from 1× to 8× the treatment volume: then the calculated doubling time of surviving clonogenic cells is 0, 7·2, 12·8 and 17·3 h if we assume that the doubling time reverts to that in untreated tumours at 1×, 2× 4× or 8× the treatment volume respectively, It may, therefore, be seen that the implied doubling time which I have calculated for the various tumours are very dependent upon whatever assumption is made regarding this factor; the data necessary to establish the truth of the situation are generally, however, not available. The *absolute* values of implied doubling times which I have deduced must therefore be viewed with considerable caution. There is, however, no doubt that wide variation in clonogenic cell doubling times do occur following different drug treatments of the same tumour, and following treatment of different tumours with the same drug.

I think that it is clear from this review that the endpoints of cell survival and tumour growth delay following chemotherapy bear a relationship to each other which is far from simple. There is no way that one can be predicted from the other without a great deal of additional information about the biology of the system and the mechanism of action of the treatment agent. The best endpoint to use for a given experiment will depend upon the objectives of the experiment, but in many cases it will be desirable to use both in order to understand as fully as possible the nature of the tumour response.

CONCLUSIONS

1. Time of assay for clonogenic cell survival is often of predominant importance and should always be taken into account when measuring tumour response. In general, 24 to 48 h after drug administration is probably the best time to carry out the assay.
2. There are a number of possible artefacts of tumour disaggregation procedures which may bias results of clonogenic cell survival. Most of these are hypothetical and their importance for specific systems is not known.
3. There are a number of systemic "host effects" of drug administration which may influence tumour growth delay.

For this reason it is desirable to avoid the use of immunogenic tumour systems.

4. For some drugs, notably adriamycin and actinomycin D, there can be a significant cytostatic effect which leads to tumour growth delay in the absence of cell killing.
5. Differences in doubling times of surviving clonogenic cells appear to occur following different drug treatments of the same tumour system.
6. Estimates of the doubling time of surviving clonogenic cells can be very dependent upon the assumptions made regarding the stage of regrowth at which the tumour reverts to control growth patterns.
7. There is no way that the response using one endpoint can be accurately predicted from the data using the other endpoint without a great deal of additional information.
8. To understand more fully the tumour response to a given agent, both endpoints should be used.

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