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Effects of Melphalan on a Sensitive and a Resistant Yoshida Ascites Tumour

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Repeated serial passage of the Yoshida ascites tumour with each innoculum of cells exposed to an alkylating agent, Melphalan (p-N, N, -di-2-chlorethyl-amino-phenyl-alanine) has produced a variant of the tumour that has approximately 100 times the resistance to the action of Melphalan compared to the original sensitive cell line. The animals bearing the sensitive tumour can be effectively cured by a single subcutaneous dose of 1 mg/kg Melphalan; the time of death of the animals bearing the resistant strain is unaffected by this dose. The mechanism of resistance is at present not understood. This paper describes an investigation of the disturbance of the cell kinetics that results from the exposure of these tumour cells to Melphalan.

The approach used has been the combined use of autoradiographic techniques to study DNA synthesis, and analytical histochemistry to determine the DNA content of single cells. The distribution of the cell population at any time amongst the various compartments of interphase (G_1, S, G_2 and M) can be resolved by this technique. The changes in the distribution of the cells in these compartments indicate the mode of action of the drug on the progress of the cells thorugh the cell cycle.

This work forms a preliminary part of an investigation into the nature of resistance to Melphalan being made in conjunction with T. A. Connors and C. R. Ball of the Chester Beatty Research Institute (detailed results will be published elsewhere).

Materials and methods

Preparation of the animals. Female, Chester Beatty strain, rats weighing 200-250 g were innoculated intraperitoneally with 2×10^4 sensitive Yoshida ascites cells (SYAC) or resistant Yoshida ascites cells (RYAC). Resistance was maintained in the RYAC line by treating the host 3 days after transplantation with 2 mg/kg Melphalan subcutaneously. The tumours were investigated on the 6th day after inoculation. The resistant line was treated in all previous passages, but not treated until the 6th day in the animals being investigated. The cell counts in the peritoneal cavity on the 6th day were approximately 8×10^4 in both cell lines.

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Isotopes. Tritiated thymidine (^{3}H-TdR), specific activity 5 C/mM, was obtained from the Radiochemical Centre, Amersham, Bucks.

Feulgen staining. Feulgen staining was carried out according to the method described by Hale (1963). The hydrolysis was for 10 minutes in NHCl at 60°C.

Autoradiography. Ilford K5 nuclear research emulsion was used for coating the slides which were then exposed at 4°C for a suitable period. Kodak D 19B was used for developing, and Amfix (May & Baker) with hardener for fixing.

Percentage labelled metaphase curves (Quastler and Sherman, 1959; Wimber, 1960). Rats were injected with ^{3}H-TdR, either intraperitoneally (0.5 μc/g body weight) or subcutaneously (1.0 μg/g body weight). 0.05 ml samples of ascitic fluid were withdrawn by syringe at intervals and transferred to 1 or 2 ml of heparinised Hanks' solution. Preparations were made in two ways. One method involved making smear preparations after the suspension had been concentrated by removal of the greater part of the supernatant following centrifugation at 1000 r. p. m. in an M.S.E. Minor centrifuge. The smears were fixed in absolute methanol. The alternative method was to wash the cells in Hanks' solution, incubate for 15 minutes in Hanks' solution diluted four times and to fix in the centrifuge tube with methanol/glacial acetic acid 3 : 1 before making drop preparations on cooled, grease-free slides. The advantage of the second method, involving the hypotonic treatment, was that the metaphases became spread and so, easily distinguishable, but there was difficulty in that the cells tended to clump. The slides were washed in tap water for 10 minutes before they were stained with Feulgen or proponic orcein stains, washed and made into autoradiographs. The percentage of metaphases that were labelled at each time of sampling was determined.

Determination of percentage labelling and mitotic index. Approximately 0.05 ml of ascitic fluid was withdrawn from the rat by syringe and incubated at 37°C for 15 minutes in 2 ml of previously warmed medium: Hanks' balanced salt solution containing 30% horse serum (Wellcome) and 2 μc/ml ^{3}H-TdR. Smear preparations were made when the cell suspension had been suitably concentrated. The smears were then fixed in absolute methanol and washed for 10 minutes in tap water. Some of them were stained with orcein and used for the determination of mitotic index by examination of 2000 cells; others were stained by the Feulgen method and made into autoradiographs. The percentage of cells labelled with ^{3}H-TdR was determined from the examination of 2000 cells.

Ascitic fluid samples were taken for these procedures from untreated sensitive and resistant rats and from rats at different times after the injection of Melphalan, 1.0 mg/kg subcutaneously.

Measurement of DNA contents of individual cells. The DNA contents of individual cells were determined by measurement of Feulgen absorbance at 5500 Å using a Deeley pattern integrating microdensitometer (Deeley, 1955).

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Compartment analysis. Measurement of the DNA contents of 100 randomly selected unlabelled cells in a Feulgen stained autoradiograph made it possible to estimate the distribution of the unlabelled cells between G_1 and G_2. This information, together with the labelling index and the mitotic index, was used to estimate the percentage of the total population to be found in each compartment of the cell cycle.

Correlation of intensity of labelling of S cells with DNA content. Photographic maps were made of representative areas of Feulgen stained and autoradiographed smears of untreated sensitive and resistant cells. The autoradiographic exposure time had been chosen to give a very high grain density in the cells which had taken up most tritiated thymidine. Grain densities of the labelled cells were recorded as low, medium or high, low corresponding to 15 grains or less, medium corresponding to higher but still countable grain number, and high corresponding to a density where there was coalescence of grains. The grains were then removed by potassium ferricyanide and photographic fixer as previously described (Balfour *et al.*, 1965) and the DNA contents of the DNA synthesising cells were measured.

Demonstration of macrophages by carbon uptake. Colloidal carbon. 64 mg/ml (Halpern, *et al.*, 1953) was diluted to 6.4 mg/ml with physiological saline. 0.5 ml of the suspension was injected intraperitoneally and after approximately an hour smears were made in the standard way, fixed in methanol and stained with a Romanowsky stain.

Results

The overall growth rate of the ascites cells was estimated by washing out the peritoneal cavities of rats on different days after implantation and counting the total cells present. This method gave a doubling time of approximately 18 hours for both strains. Percentage labelled metaphase curves from six untreated animals are shown in Fig. 1. Animals a-d had sensitive tumours. Animals a and b were given the dose of ^{3}H-TdR intraperitoneally, animals c and d subcutaneously. Animals e and f had resistant tumours and were given the ^{3}H-TdR intraperitoneally. From these curves the mean duration of $G_2 + \frac{1}{2}M$ is estimated to be approximately $3\frac{1}{2}$ hours. There is marked variation in the latter part of the curves which is suggestive of considerable differences in the individual cell cycle times.

It was noted that there was a wide range in the intensity of tritium labelling of cells in DNA synthesis whether the labelling had taken place *in vivo* or *in vitro*. It was shown that there was no correlation between intensity of labelling and DNA content at the time of labelling.

No significant difference was shown between the mitotic indices of untreated ascites cells of the two lines (20 sensitive animals, 10 resistant but the untreated resistant tumours showed a slightly higher percentage labelling than the untreated sensitive tumours ($P = 0.05$).

Measurement of the DNA contents of 600 sensitive cells not labelled with ^{3}H-TdR and of the same number of unlabelled resistant cells confirmed the finding based on

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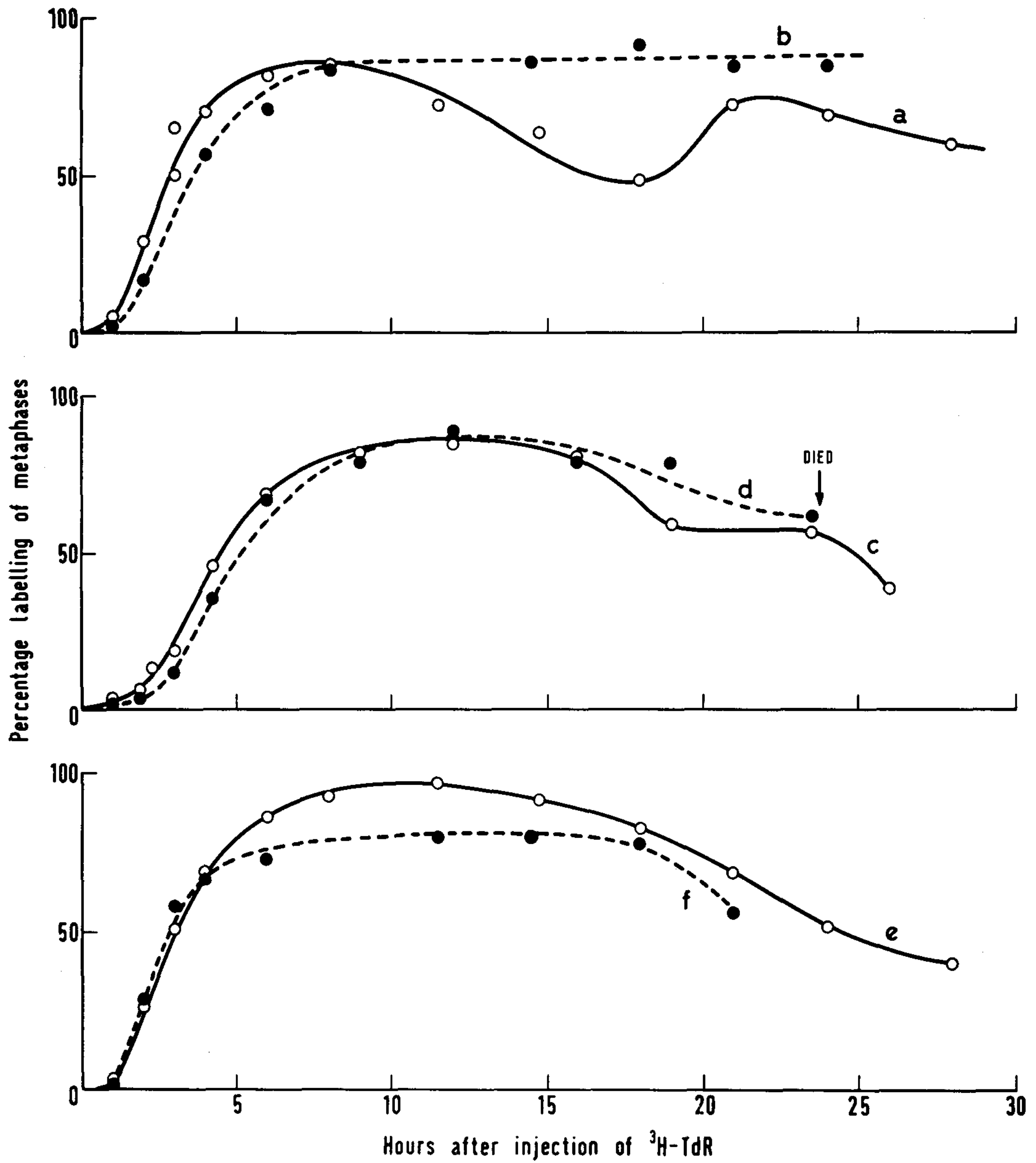

Fig. 1. Percentage labelle mitosis curves for SYAC a-d, and RYAC e and f

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chromosome analysis (Ball *et al.*, 1966) that the cell lines show little aneuploidy (Fig. 2). Polyploid cells formed less than 1% of the population and only approximately 1% of the population had DNA contents suggesting that the cells were in DNA syn-

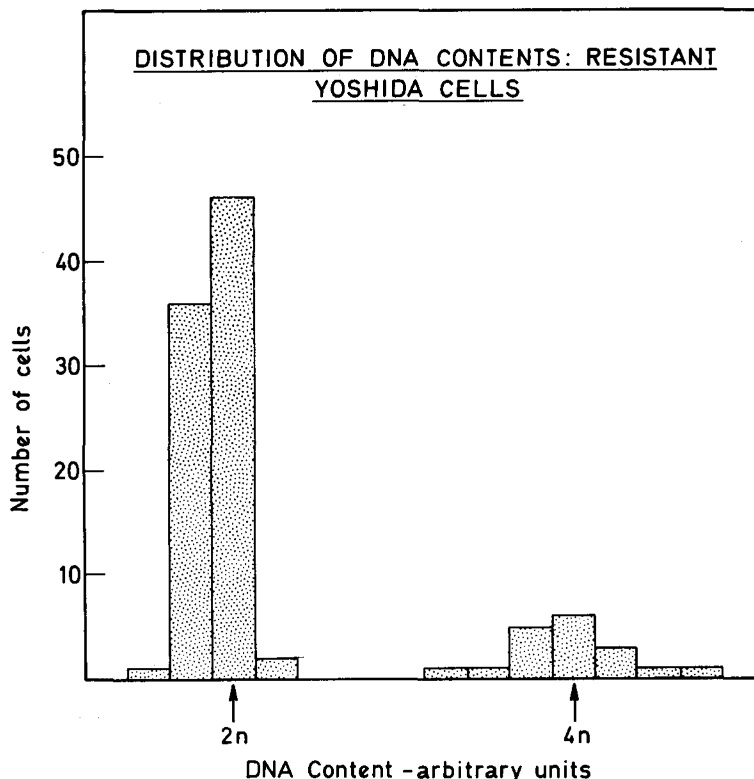

Fig. 2. Distribution of DNA contents of a sample of the unlabelled cells (i.e., G_1 and G_2) in a RYAC population

thesis when the cells did not appear to be labelled. The mean size of the G_2 compartment was higher in the untreated SYAC than in the RYAC (Tab. 1).

After the treatment with Melphalan there was a continuous fall in the mitotic index of the sensitive cells preceding the decrease in total cell number. Few mitoses were to be seen at 48 hours, and of these the majority were of an abnormal type, which might not be expected to complete effective division. The resistant cells showed only a small fall in mitotic index ($P = 0.05$) followed by recovery to normal (Figs. 3 and 4).

In both lines, following treatment with Melphalan, there was a small but significant fall in percentage labelling followed by a rise (Fig. 5 and 6). (Fall: sensitive, $P = > 0.005$; resistant, $P = > 0.01$. Rise: sensitive, $P = 0.001$; resistant, $P = > 0.001$). In the calculation of the significance of the rise in percentage labelling of

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Tab. 1. Compartment analysis - Percentage of cells in each compartment

Hours after treatment	N	Sensitive				N	Resistant			
		G_1	S	G_2	M		G_2	S*	G_2	M
	(6)	42.3	40.3	13.1	2.0	(6)	46.8	42.5	8.2	2.3
12 hours	(2)	44	35.5	20.5	0.5	(2)	52.7	38.5	8.8	1.4
17-25 hours	(4)	20.8	54	25.2	*	(4)	31.7	57	11.2	1.5
48	(1)	17	5.3	71.8	0.3		—	—	—	—
72	(1)	10.4	7.5	72.2	0.5		—	—	—	—

Controls in italics
Number of observations in brackets
* Wide variation between <0.5 — 0.1%
S and M % were determined on 2000 cells
G_1 and G_2 were determined on 100 cells

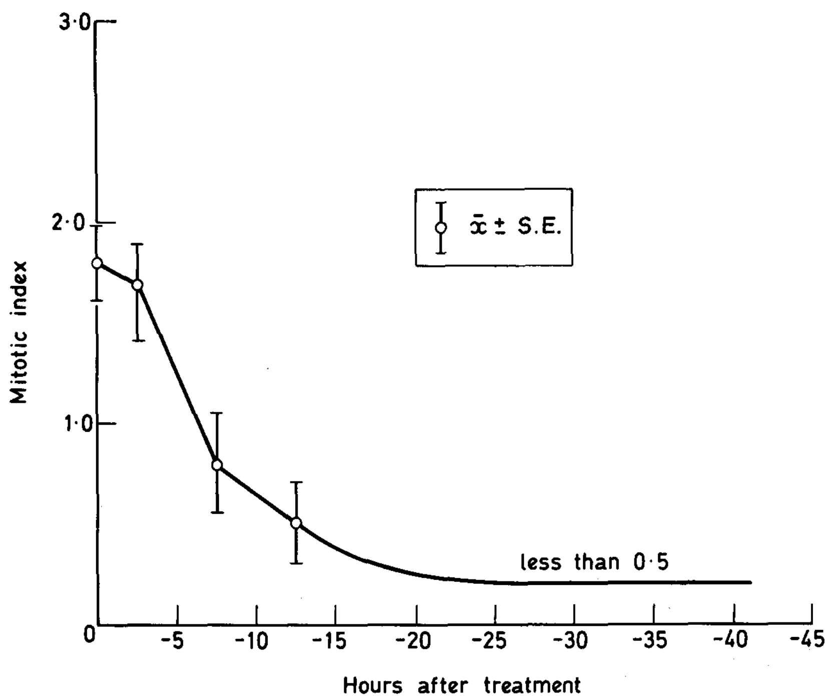

Fig. 3. Mitotic index of the SYAC following treatment with Melphalan 1 mg/kg, subcutaneously

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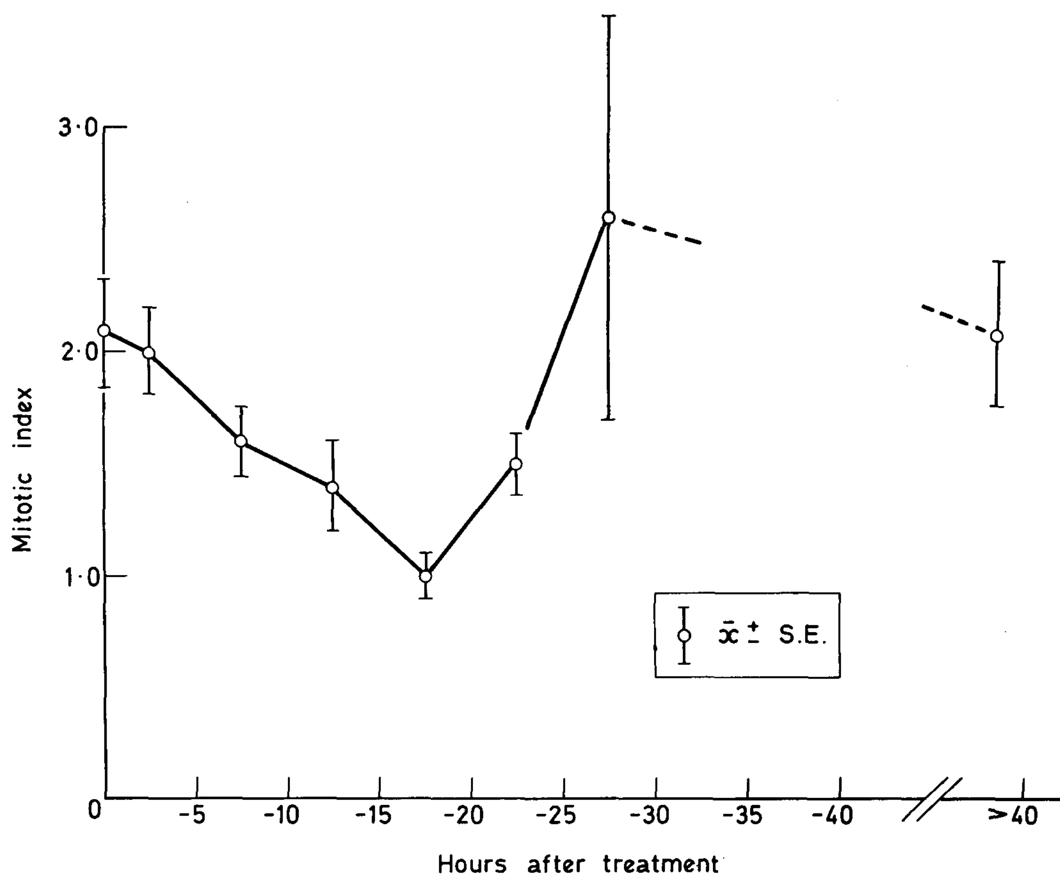

Fig. 4. Mitotic index of the RYAC following treatment with Melphalan 1 mg/kg, subcutaneously

the resistant line, one result was excluded whose value was approximately one third of the mean value of the other four results. The result was, however, included in the calculation of the standard error shown.

It was apparent that the mean grain count fell in both the sensitive and resistant ascites tumours after treatment with Melphalan but no attempt was made to quantitate the observation owing to the very wide range in the number of grains per cell.

During the period after Melphalan administration in which growth was arrested, the G_2 compartment increased in size and the size of the G_1 compartment decreased. These changes took place in both lines but were more marked in the sensitive cells (Tab. 1). When cell death became appreciable the situation became complex and at times later than 48 hours macrophages as identified by their carbon uptake formed a significant part of the population. Because of these factors most a significant part

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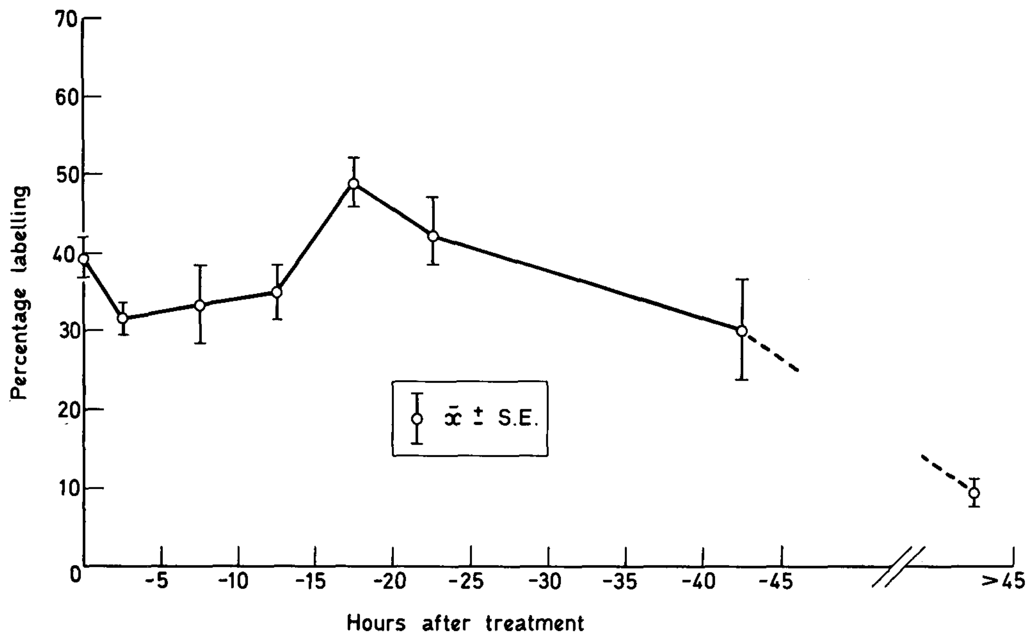

Fig. 5. Percentage labelling with ³H-TdR in the SYAC following treatment with Melphalan 1 mg/kg subcutaneously

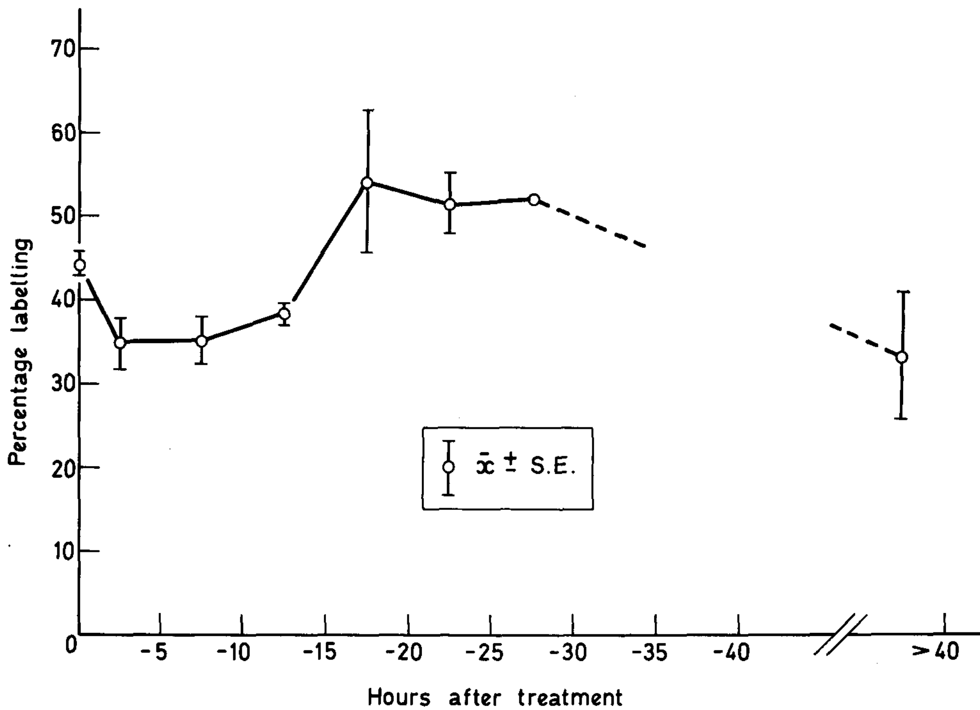

Fig. 6. Percentage labelling with ³H-TdR in the RYAC following treatment with Melphalan 1 mg/kg subcutaneously

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of the population. Because of these factors most of the compartmental analyses were performed during the first 25 hours after treatment, but an analysis of sensitive cells at 48 hours and another at 72 hours showed the majority of the cells in G_2. Resistant animals at this time had died or were dying. Coincidental with the growth of the ascites cells, secondary tumours developed in the omentum. Histological examination of this tumour showed a marked cytolysis and karyorrhexis at 24 hours in the sensitive line. Light and electron microscopic examination of the resistant solid and ascitic cells failed to show any lesions 24 and 48 hours after treatment.

Discussion

The Yoshida tumour has a predominantly uni-modal chromosome number; the DNA measurements have confirmed that only a very few unlabelled cells are to be found between the 2c and 4c modes. Hence, as aneuploidy is an unimportant feature of this system, changes in the relative numbers of unlabelled cells at the 2c and 4c modes can be ascribed to changes in the relative numbers of cells in the G_1 and G_2 compartments of interphase. On the other hand, it is clear that the cell population has a considerable range of individual cell cycle times, though the overall doubling time for the tumour mass is fairly constant. This variation of cell cycle times would tend to mask some of the more subtle changes in the cell kinetics, though as can be seen from the results the major changes induced by giving the drug can be resolved by the technique that has been used.

As several different changes in the cell proliferation pattern are occurring simultaneously, it is of some value to summarize the main events for each tumour line.

The SYAC showed during the first twelve hours after treatment a rapid fall in the mitotic index and a build up of cells in the G_2 compartment, the percentage of cells in S being a little reduced by the end of this period. At this time no significant change had taken place in the total number of cells present or in their morphology, as seen in the light microscope. By the end of the first day after treatment, the mitotic index had fallen to very low limits and abnormal mitoses were present. The arrest of cells in G_2 was still present and the percentage of cells in S had risen, but the low grain counts would suggest that this increase of the cells in S was an outcome of the duration of the S period being increased as the result of a slower rats of DNA replication. During the next 48 hours, there was a marked decrease in the number of tumour cells; preparations showing widespread evidence of cell destruction were seen at this time. The relatively few cells surviving at 72 hours were mainly arrested in G_2. It is evident that the rapid dissolution of the SYAC cannot be accounted for by virtue of the mitotic inhibition alone. Interphase death is the only explanation that would account for the vast tumour destructive effects of the drug. These experiments indicate that there is a disturbance of the cell kinetics prior to the cell killing phase, but they give no indication that there is a predilection for cell death to occur in any one specific interphase compartment.

The RYAC showed some perturbation of their kinetics when treated with Mel-

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phalan. The mitotic index and the percentage labelling both fell during the first 24 hours after treatment. It was noticeable that the G_2 arrest seen in the SYAC was absent in the RYAC strain.

The period following the first 24 hours after treatment in the animals bearing the RYAC was difficult to analyse as the death of the animal due to the effects of the tumour occurred at this time. However, some information is available. The depressed mitotic index recovered to normal limits with a possible overshoot at about 25-30 hours. The percentage of labelled cells (cells in S) was a little increased above normal during this time. This suggests that the resistant cells are probably temporarily affected by the alkylating agent but have a rapid and effective mechanism for restoring the damage and reverting to their previous high rate of proliferation.

The work described in this paper is still in progress and a number of parallel biochemical investigations have been made; it is for this reason that we wish to postpone the full discussion of this problem until all the facts are available.

The investigation has indicated that the combination of autoradiography with microdensitometry may be a valuable approach for the direct analysis of changes induced in interphase cells as the result of contact with alkylating agents.

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Summary

The mode of action of the alkylating agent Melphalan on Yoshida ascites tumours resistant and sensitive to alkylating agents in rats has been studied. A combination of autoradiography and analytical histochemistry has been used to measure changes in the cell kinetics. The sensitive strain showed a marked fall in mitotic index, build up in G_2 and finally interphase death occurring 24-72 hours after treatment. The resistant form showed only slight mitotic inhibition and a possible recovery phase 24-30 hours after treatment, without evidence of cytological damage.

References

Balfour B. M. *et al.* (1965). DNA metabolism of the immunoglobulin containing cells in the lymph nodes of rats. *J. Reticuloendothel. Soc.*, **2**: 379.

Ball C. R. *et al.* (1966). Comparison of nitrogen mustard sensitive and resistant Yoshida sarcomas. *Int. J. Cancer*, **1**: 319.

Deeley E. M. (1955). An integrating microdensitometer for biological cells. *J. Sci. Instrum.*, **32**: 263.

Hale A. J. (1963). The leucocyte as a possible exception to the theory of DNA constancy. *J. Path. Bact.*, **85**: 311.

https://doi.org/10.1017/S1120962300026275 Published online by Cambridge University Press

HALPERN B. N. *et al.* (1953). Quantitative study of granulopectic activity of reticuloendothelial system. I- The effect of the ingredients present in India ink and of substances affecting blood clotting *in vivo* on fate of carbon particles administered intravenously in rats, mice and rabbits. *Brit. J. Exp. Path.*, **34:** 426.

QUASTLER H., SHERMAN F. G. (1959). Cell population kinetics in intestinal epithelium of the mouse. *Exp. Cell Res.*, **17:** 420.

WIMBER D. E. (1960). Duration of the nuclear cycle in Tradescantia paludosa root tips as measured with H^3 -thymidine. *Amer. J. Bot.*, **47:** 828.

RIASSUNTO

È stato studiato il meccanismo d'azione, nel ratto, dell'agente alchilante Melphalan sui tumori ascite Yoshida, sia resistenti che sensibili agli agenti alchilanti. Per la misurazione dei cambiamenti nella cinetica cellulare è stata usata una combinazione di autoradiografia e istochimica analitica. Il ceppo sensibile ha presentato un notevole abbassamento dell'indice mitotico, aumento di G_2 ed in ultimo morte in interfase 24-72 ore dopo il trattamento. La forma resistente ha presentato soltanto inibizione mitotica ed una fase di possibile ristabilimento 24-30 ore dopo il trattamento, senza evidenti danni citologici.

RÉSUMÉ

Le mécanisme d'action du Melphalan sur les tumeurs ascites Yoshida, résistentes ou sensibles aux agents alchylants, a été étudié chez le rat. Pour mesurer les changements dans la cinétique cellulaire, l'autoradiographie a été combinée à l'histochimie analytique. La forme sensible a présenté une remarquable diminution de l'index mitotique, une augmentation de G_2 et, finalement, mort en interphase 24-72 heures après le traitement. La forme résistante n'a présenté qu'une inhibition mitotique et une phase de possible rémission 24-30 heures après le traitement, sans dommages cytologiques évidents.

ZUSAMMENFASSUNG

Untersuchung über den Wirkungsmechanismus des Alkyle-wirkstoffs Melphalan bei den Aszitessarkomen (Yoshida) der Ratte, die auf Alkylewirkstoffe ansprachen oder resistent waren.

Zur Messung der Veränderungen in der Zellkinetik wurde eine Kombination von Autoröntgenographie und analytischer Histochemie angewandt. Bei den auf Alkylewirkstoffe ansprechenden Tumoren zeigte sich eine merkliche Minderung des Mitoseindexes, Anstieg von G_2 und schliesslich Absterben der Tiere in der Interphase 24-72 Stunden nach der Verabriechung. Bei der resistenten Form zeigte sich lediglich eine Mitoseinhibierung und 24-30 Stunden nach der Verabreichung eine für die Wiederherstellung günstige Phase, ohne deutliche Zellschäden.

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