to half size between the 1st and 9th days post wounding. In X-irradiated animals, wounds of conventional animals fell from peak size to 1.8 on the 4th post wounding day to 0.9 on the 13th day; wounds of germfree rats from a peak of 1.2 on the 2nd post wounding day to 0.6 on the 10 th day post wounding.

The effect of the microbial flora on wound healing has been the subject of sporadic investigation and often conflicting results. ROVIN et al.[9], CARTER et al.[10] and BRODY et al.[11] were unable to demonstrate any change in healing rate, granulation tissue or rate of collagen production in germfree or conventional animals. The present study supports these findings utilizing the open wound contraction model of GRILLO[7] which eliminates problems inherent in using strength of wound as an index of healing.

In the present study irradiation did not produce a retardation in the germfree animal where there has been elimination of uncontrolled and variable bacterial infection as a complicating factor of the surgical repair mechanisms. It should be pointed out that there are other differences in the gnotobiotic animals including a diminished total lymphocyte mass and immature immunologic capacity and resultant, increased radiation resistance. Bacterial contamination which occurs following radiation injury in non-gnotobiotic animals provides a partial explanation for the lack of retardation of the wound closure pattern observed in the germfree animal. These observations suggest that wound healing abnormalities which occur following radiation in rats result, in part, from bacterial contamination and entry of more virulent organisms due to impaired immune capability secondary to hematologic radiation injury.

Zusammenfassung. Es wurde die Wirkung von Röntgenbestrahlung (800 R) am Heilungsprozess bei offenen Wunden in normalen und keimfreien Ratten untersucht. Die Bestrahlung verlangsamte die Wundheilung bei den normalen, jedoch nicht bei den keimfreien Ratten.

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The Effects of Low Concentrations of Actinomycin D upon Nucleic Acid Synthesis in Different Cell Types

An important property of actinomycin D (AMD) is to bind to the DNA molecule in mammalian cells[1]. At certain doses AMD has been found to delay initiation of DNA synthesis in Ehrlich ascites cells and mouse jejeunum in vivo[2]. This effect has also been observed in vitro at concentrations within the range 0.01–0.1 μg/ml[3,4]. Within this range of concentration, AMD appears to inhibit selectively nucleolar synthesis of RNA without having any immediate effect on either the rate of DNA synthesis or on RNA synthesis in extra-nucleolar parts of the nucleus[4–6].

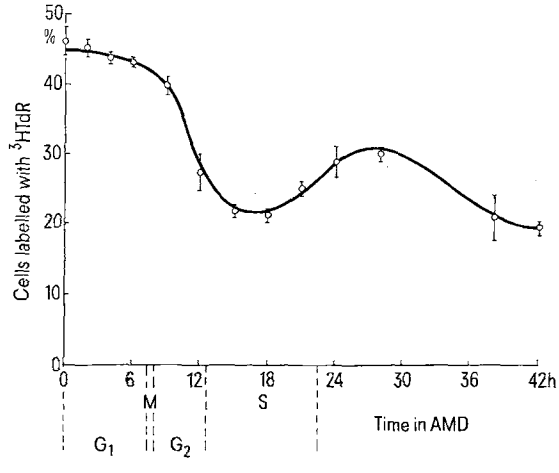

Fig. 1. Percentage of an asynchronous culture of L-cells labelled by a pulse exposure to ^{3}HTdR as a function of time in AMD. ○, 0.04 μg/ml.

RICKINSON[4] has shown that continuous incubation of an asynchronous population of L-cells with 0.04 μg/ml AMD blocks entry of cells into the DNA synthetic phase after about 6 h. The blockage is incomplete however, and some 12 h later a substantial percentage of the population succeeds in entering S phase and completes another round of DNA synthesis. In recent work on the initiation of DNA synthesis, HATFIELD et al.[7] did not observe this effect when either embryonic mouse fibrolasts (EMF) or HEp/2 cells were continuously incubated with 0.04 μg/ml AMD. The present experiments were therefore undertaken to confirm RICKINSON's result and to determine if the effect is peculiar to mouse L-cells or if it can be demonstrated for other cell types by changing the concentration of AMD.

Materials and methods. Both mouse L-cells and HEp/2 cells were used as established cell lines in these experiments. These cells were grown in Pyrex feeding bottles with medium 199 (Wellcome Ltd), and 10% foetal calf serum (Flow Laboratories), for L-cells, and Eagle's medium (Wellcome Ltd.) and 10% foetal calf serum buffered to pH 7.2 with bicarbonate for HEp/2 cells.

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Monkey kidney (MK) and embryonic mouse fibroblast (EMF) cultures were used as primary cell lines. MK cells were obtained as primary cultures and were grown in medium 199 with 10% foetal calf serum and 5% lactalbumin hydrolysate. EMF cultures were prepared by standard methods[8] and were grown in the same medium as MK cells. Both MK and EMF cells were used for experiments in the second passage. For experimental work coverslips were seeded with 5×10^4 cells and incubated at 37 °C under 5% CO_2 – 95% air for at least 24 h before addition of drug.

A commercial preparation of AMD (Merck Sharpe & Dohme Ltd.) was dissolved in isotonic saline at pH 6.0 to give a stock solution at 4×10^{-6} g/ml. The solution was stored at –20 °C and was used for experiments when not less than 1-day-old and not more than 1-month-old.[9]. After the cultures had been treated with different concentrations of AMD further diluted in feeding medium, for different time intervals, the cells were exposed to medium containing either ^{3}H-thymidine (TdR) (5 µCi/ml, 5 Ci/m*M*) or ^{3}H-uridine (UdR) (20 µCi/ml, 24 Ci/m*M*), for 15 min. The cultures were then rinsed twice in Ringer, fixed in acetic acid-ethanol (1:3) for 15 min, rinsed in 70% ethanol for 5 min, hydrolysed with 2% perchloric acid at 4 °C for 2 periods of 30 min to remove label not bound into nucleic acid and rinsed thoroughly with water.

Labelled coverslips were mounted on to gelatinized slides and dipped into diluted 1:1 Ilford K-2 emulsion. The autoradiographs were developed after 3 days for ^{3}H-TdR and 2 days for ^{3}H-UdR and the cells were stained with Giemsa. Average values for the grain count per labelled cell were obtained from counts on 30 labelled cells per coverslip, the percentage of ^{3}H-TdR labelled cells was obtained from counts on 1,000 cells per coverslip.

Results. Figure 1 shows the percentage of L-cells labelled with ^{3}H-TdR in a random population following increasing lengths of time in medium containing 0.04 µg/ml AMD. The percentage of cells in S phase decreased between 6 and 18 h, but increased again from 18 h to 28 h. After 28 h a progressive reduction in the percentage of labelled cells was observed.

In Figure 1 and in Figures 2–4 to be described, the duration of the phases of the cell cycle have been shown for each cell type. The data is taken from HATFIELD et al.[7] and in each case has been presented so that the time spent in AMD before cells in a particular phase reached the next $G_1 \rightarrow S$ transition, assuming undisturbed progress, can be read directly from the time axis.

Figure 2 shows the fraction of HEp/2 cells labelled with ^{3}H-TdR in a random population following increasing lengths of time in medium containing 4 different concentrations of AMD within the range 0.04–0.4 µg/ml. The effect of 0.04 µg/ml AMD is different from the effect on L-cells. 24 h treatment at this concentration caused no measurable decrease in the percentage of labelled HEp/2 cells which only began to fall after 24 h and then fell steadily. An early reduction in the percentage of labelled cells could be effected by increasing the concen-

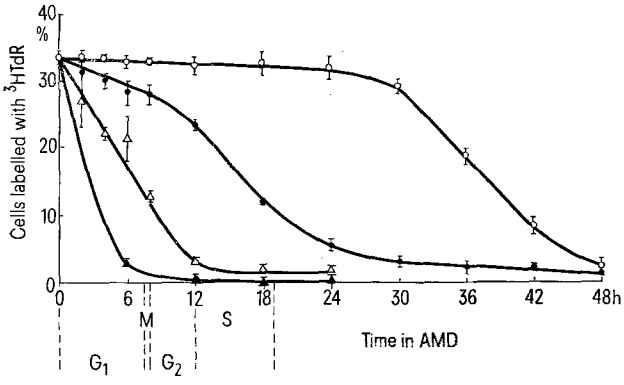

Fig. 2. Percentage of an asynchronous culture of HEp/2 cells labelled by a pulse exposure to ^{3}HTdR as a function of time in AMD. ○, 0.04 µg/ml; ●, 0.07 µg/ml; △, 0.1 µg/ml; ▲, 0.4 µg/ml.

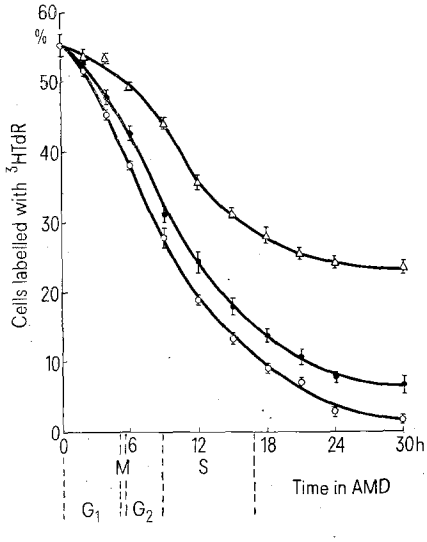

Fig. 3. Percentage of an asynchronous culture of MK cells labelled by a pulse exposure to ^{3}HTdR as a function of time in AMD. △, 0.01 µg/ml; ●, 0.02 µg/ml; ○, 0.04 µg/ml.

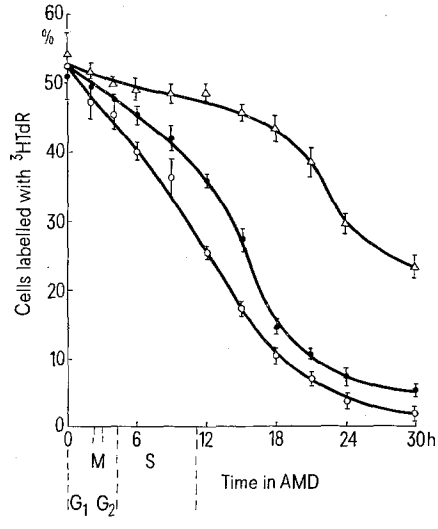

Fig. 4. Percentage of an asynchronous culture of EMF cells labelled by a pulse exposure to ^{3}HTdR as a function of time in AMD. △, 0.01 µg/ml; ●, 0.02 µg/ml; ○, 0.04 µg/ml.

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tration of AMD but it was not possible to find a dose at which cells were released into S phase after an initial block.

The fraction of an asynchronous population of MK cells labelled with ^{3}H-TdR following increasing lengths of time in medium containing 3 different concentrations of AMD within the range 0.01–0.04 μg/ml is shown in Figure 3. The percentage of labelled cells started to decrease between 2 and 6 h after the addition of AMD depending on drug concentration and then decreased steadily.

Figure 4 shows the fraction of a population of asynchronous EMF cells labelled with ^{3}H-TdR following increasing lengths of time in medium containing the same concentrations of AMD as those used for MK cells. The results obtained for EMF cells were almost identical to those for MK cells at all 3 concentrations, even though the duration of the phases in the cell cycle are very different.

The effect of 0.04 μg/ml AMD on the synthesis of very different nucleolar RNA, and RNA synthesis in the extranucleolar parts of the nucleus of L-cells is shown in Table I. As stated in the introduction, a 2 h exposure to this concentration of AMD affects nucleolar RNA synthesis without significant reduction in nucleoplasmic RNA synthesis. Treatment with the same concentration of AMD for 14 or 24 h caused the nucleolar structure to disappear morphologically. Therefore only the grains over the total nucleus could be counted at these times. The number of grains over the nuclei of treated cells was reduced at both 14 and 24 h when compared with the total nuclear count over control cells but relative to the nucleoplasmic count there was no significant reduction until 24 h.

Table II shows the effects on RNA metabolism in HEp/2 cells of increasing lengths of time in medium containing 3 different concentrations of AMD within the range 0.04–0.1 μg/ml. 2 h exposure at 0.04 and 0.07 μg/ml caused a reduction in nucleolar RNA synthesis without any significant decrease in nucleoplasmic RNA synthesis. 2 h treatment at 0.1 μg/ml AMD did cause a slight reduction in nucleoplasmic RNA synthesis, but caused a much greater reduction in nucleolar synthesis of RNA. Longer exposures than 2 h again caused nucleolar disintegration at all concentrations, and the number of grains over the whole nucleus of HEp/2 cells was reduced by treatment with all 3 concentrations of AMD after 14 and 24 h.

Table I. The effects of actinomycin D upon ^{3}H-uridine incorporation into L-cells

Dose of AMD	Time in AMD (h)	Grain count		
		Nucleolus	nucleoplasm	Total nucleus
0.04 μg/ml	0	24.6 ± 2.5	38.9 ± 3.0	63.5 ± 4.0
	2	10.7 ± 1.0	34.1 ± 3.6	44.8 ± 3.8
	14	—	—	34.7 ± 3.8
	24	—	—	22.1 ± 1.3

Table II. The effects of actinomycin D upon ^{3}H-uridine incorporation into HEp/2 cells

Dose of AMD	Time in AMD (h)	Grain count		
		Nucleolus	nucleoplasm	Total nucleus
0.04 μg/ml	0	9.7 ± 2.1	19.2 ± 2.9	28.9 ± 4.4
	2	3.7 ± 0.7	16.8 ± 2.4	20.5 ± 4.3
	14	—	—	9.2 ± 0.7
	24	—	—	5.8 ± 1.3
0.07 μg/ml	2	2.9 ± 0.6	16.1 ± 1.8	19.0 ± 1.6
	14	—	—	5.4 ± 0.7
	24	—	—	3.8 ± 0.5
0.1 μg/ml	2	0.8 ± 0.4	7.3 ± 1.0	8.1 ± 1.4
	14	—	—	4.0 ± 0.4
	24	—	—	2.9 ± 1.1

Discussion. The work confirms the observations of RICKINSON[4] that when an asynchronous culture of mouse L-cells is incubated continuously with 0.04 μg/ml AMD, many of the cells which are initially prevented from entering S phase subsequently succeed in doing so. Studies with HEp/2, EMF and MK cells show that if a block forms in the G_1 phase none of these cell types is able to escape into S phase for any concentration of AMD at which the effect might reasonably be expected to occur.

It can therefore be concluded that after the initial block has developed at about 6 h, biochemical processes essential for the entry of the cell into the next DNA synthetic phase are occurring in L-cells possibly at a reduced rate, during continuous incubation with 0.04 μg/ml AMD. By 6 h however, these processes seem to have been prevented for EMF and MK cells. Conversely, for HEp/2 cells they appear to be very little affected.

Progressive resistance to 0.04 μg/ml AMD from MK and EMF cells, through L-cells to HEp/2 cells has been reported by HATFIELD et al.[7], but this paper shows that the release of cells into S phase some time after a block has formed is characteristic to L-cells and cannot be demonstrated with the other cell types. These results suggest that a careful comparison of the biochemical processes occurring in these 4 cell types between 5 and 18 h after the addition of 0.04 μg/ml AMD, possibly using synchronous cultures where feasible, might give further useful information on those processes which are essential for the continued initiation of DNA synthesis.

Results in Tables I and II are further evidence that gross forms of RNA synthesis are unlikely to be critically important in the $G_1 \rightarrow S$ transition[7]. Between 14 h and 24 h after the addition of AMD, ^{3}H-uridine uptake into HEp/2 cells is, if anything, more inhibited than uptake into L-cells, and this is quite contrary to the effect on the percentage of cells in S phase at this time[10].

Résumé. L'effet des concentrations faibles de l'actinomycine D sur le pourcentage de cellules dans la phase S pendant un contact de longue durée a été étudié dans 4 souches de cellules. Les résultats montrent que le blocage qui a lieu à la fin de G_1 n'est que temporaire pour les cellules L-strain, mais qu'il dure plus longtemps pour HEp/2, les fibroblasts de la souris et les cellules du rein du singe.

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