Psoralen Photosensitization of L 1210 Leukaemia Cells: an Approach to a New Combined Therapy

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Psoralen photosensitization of L 1210 cells has a strong effect on DNA and RNA syntheses and this result appears connected with the psoralen photobinding to DNA. Protein synthesis is less sensitive and its inhibition seems due to a different photochemical interaction, very likely to the psoralen photobinding to RNA.

A combined therapy using cyclophosphamide and L 1210 cells psoralen-photoinactivated was performed after the leukaemia transplant, showing a significant decrease in mortality, even in comparison with the simple treatment with the alkilating drug.

Introduction

Furocoumarins are a group of well-known photosensitizing drugs that by irradiation with long wavelength ultraviolet light react with the pyrimidine bases of DNA forming C_4-cyclo-adducts [1, 2].

In a living cell the main consequence of furocoumarin photosensitization is the block of cell replication [3, 4]; in fact, furocoumarins can kill very different organisms such as bacteria [5], viruses or mammalian cells [2, 3].

Particular aspects are shown by photosensitization of tumor cells; in previous papers, we dealt with this problem using two experimental mouse tumors, the Ehrlich ascites and the Graffi leukaemia cells. In both cases, after psoralen photosensitization, in addition to a strong inhibition of macromolecular synthesis [6, 7], the loss of cell replication, *i. e.* the capacity to transmit the tumor by transplant, was observed [4, 8].

Moreover, the injection of the thus photoinactivated tumor cells succeeded in protecting the mice from a subsequent challenge performed with the same but viable tumor cells [8, 9].

With the aim of obtaining further and more satisfactory data about the possible employment of tumor cells inactivated by furocoumarins in cancer immuno-therapy, we have now studied, using the L 1210 leukaemia, the possibility of performing a combined therapy after the leukaemia transplant by treatment with an alkilating drug and cells photoinactivated by psoralen.

However, before this, it was necessary to study some aspects, not yet investigated, of psoralen photosensitization of these leukaemia cells.

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Materials and Methods

Chemicals

Psoralen was prepared by chemical synthesis [10]. [^{3}H]thymidine, specific activity 19 Ci/mM, [^{3}H]uridine, specific activity 25 Ci/mM, and an equimolecular mixture of fourteen [^{14}C]amino acids, specific activity 10 mCi/mM, were purchased from the Radiochemical Centre, Amersham, England. Actinomycin D, obtained from Chalbiochem, was dissolved in ethanol 50% (250 μg/ml). Cyclophosphamide, obtained from Schering, was dissolved in saline solution.

L 1210 leukaemia

The L 1210 leukaemia was routinely transferred by injecting the leukaemia cells into $DBA/_2$ mice, 10^5 cells/mouse; after 5–6 days, this injection produced an exudate of about 2 ml and killed all the animals within 7–8 days.

All solutions or cell suspensions were injected in a uniform volume of 0.2 ml/mouse.

In the therapy experiments, while the treated mice received drugs or inactivated cells, the control mice were injected with saline solution.

Irradiation procedure

5 ml of cell suspension of L 1210 leukaemic cells in balanced saline solution (2×10^6 cells/0.1 ml) containing psoralen (20 μg/ml) were poured into a

Petri dish, 5 cm in diameter, chilled on crushed ice and irradiated by a Philips HPW 125 lamp. In these conditions the incident radiation on the whole sample corresponded to 2.02×10^{16} quanta/sec.

DNA, RNA and protein syntheses

The irradiated cells were washed with ice-cold balanced saline solution and then incubated at 37 °C in Hank's solution (4×10^6/0.1 ml) in the presence of the suitable radioactive precursor [3 μCi/ml of [^{3}H]thymidine (for DNA) or of [^{3}H]uridine (for RNA); 0.5 μCi/ml of mixture of [^{14}C] amino acids (for proteins)].

To study the DNA and RNA syntheses, after 30 min of incubation, the nucleic acids were extracted by the hot 10% sodium chloride method [6] and their specific activity determined using a modified Bray's fluid and the diphenylamine [11] or orcinol [12] reactions.

To study the protein synthesis [7], aliquots containing about 1 mg of proteins [evaluated according to Lowry [13]] were removed at fixed times from the incubation mixture, precipitated with 5% trichloroacetic acid, collected on Whatman GF/C filters and counted by using a toluene-based solution.

These experiments were generally carried out as follows:

a) Samples of cell suspension containing psoralen were irradiated for increasing times;
b) samples of cell suspension without psoralen were irradiated for the same times;
c) a sample of cell suspension containing psoralen was kept in the dark.

Irradiation in the absence of the drug, as well as incubation in the dark in its presence, were both ineffective; inhibition of macromolecular synthesis occurred only by irradiation in the presence of the psoralen.

Radioactivity measurements

Measurements were performed by means of a Packard, mod. 3375 liquid scintillation spectrometer, using the following scintillating fluids:

a) Modified Bray's fluid: PPO 8 g, POPOP 0.2 g, naphthalene 80 g, toluene 110 ml, ethoxyethanol 110 ml, dioxane up to 1000 ml of solution.
b) Toluene-based scintillator: PPO 5 g, POPOP 0.5 g, toluene up to 1000 ml of solution.

In the double-labeling experiments, pre-select narrow windows were used, with a ^{14}C spillover lower than 10% and care was taken so that ^{3}H-counts were about ten times higher than ^{14}C-counts. In such a manner less than 1% of counts monitored by red channel was due to ^{14}C-radioactivity.

Results

DNA, RNA and protein syntheses

Fig. 1 shows the results obtained by studying DNA, RNA and protein synthesis in L 1210 leukaemia cells irradiated in the presence of psoralen.

Both DNA and RNA syntheses were strongly inhibited, practically in the same degree; the D_{37} dose (*i. e.* the ultraviolet radiation dose that in the presence of 20 μg/ml of psoralen reduced the incorporation of the radioactivity to 37% of the controls) was 5.1×10^{18} quanta for DNA synthesis and 5.6×10^{18} quanta for RNA synthesis.

Protein synthesis was also affected by psoralen sensibilization, but to a lower degree, with a D_{37} dose of 1.7×10^{19} quanta.

RNA and protein syntheses were also studied simultaneously in the same samples; leukaemia cells, irradiated in the presence of psoralen, were incubated at 37 °C in the presence of a mixture of [^{3}H]uridine and of [^{14}C]amino acids, and processed according to the method described above for protein synthesis.

The results show clearly that the incorporation of tritium radioactivity into acid-insoluble fraction was more strongly inhibited than that of ^{14}C. For example, after 8 min of irradiation, the inhibition observed was 67% for [^{3}H]uridine and 26% for [^{14}C]amino acids.

The psoralen effect on protein synthesis was also studied in the presence of a specific inhibitor of RNA synthesis, such as actinomycin D.

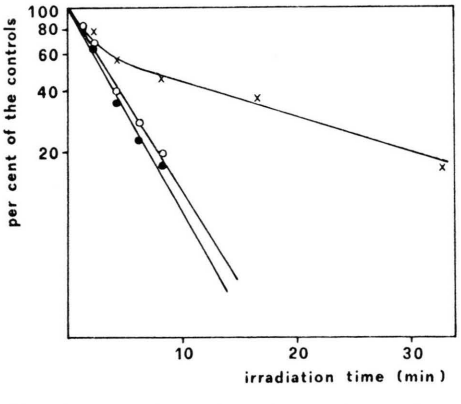

Fig. 1. Nucleic acid and protein syntheses in L 1210 leukaemia cells after irradiation in the presence of 20 μg/ml of psoralen. The irradiated cells were incubated at 37 °C in the presence of a suitable labeled precursor and the radioactivity incorporated into the macromolecules was determined. Synthesis of DNA –●–●–, RNA –○–○–, proteins –×–×–.

A sample of leukaemia cells irradiated for 8 min in the presence of psoralen was split into two parts; to the first actinomycin D was added (final concentration 5 μg/ml), the second was treated only with the same amount of the solvent. As a control a sample of untreated cells was processed in the same way. Then all the samples were incubated for 20 min in the presence of [^{14}C]amino acids and the TCA-insoluble radioactivity was determined.

The data, summarized in Table I, show that a significant inhibition of protein synthesis was observed even in the presence of actinomycin D, *i. e.* even in the absence of RNA synthesis.

Table I. ^{14}C-amino acid incorporation into the acid-insoluble fraction in the L 1210 leukaemia cells after irradiation (8 min) in the presence of psoralen (20 μg/ml) and incubating for 20 min in the radioactive medium.

	^{14}C-cpm/mg of protein	per cent inhibition
Without actinomycin D		
untreated cells	807	—
treated cells	275	66
With actinomycin D (5 μg/ml)		
untreated cells	332	—
treated cells	185	44

L 1210 leukaemia combined therapy

First we have determined the lowest irradiation time that in the presence of 20 μg/ml of psoralen is sufficient to destroy the tumor-transmitting capacity of L 1210 leukaemia cells, that turned out to be about 15 – 20 min. In the second place we have determined the highest cyclophosphamide dose that injected i.p. into NCL mice does not induce mortality, *i. e.* about 230 μg/kg.

Therefore, in the following experiments we have always used psoralen concentrations of 20 μg/ml, irradiation times of 30 min and cyclophosphamide doses of 200 μg/kg.

The combined therapy was performed as follows: 100 mice were submitted to leukaemia transplant (5×10^3 cells/mouse); 25 animals were kept without any treatment as controls.

After 10 days the other 75 animals were injected i.p. with cyclophosphamide. 50 of these mice were then injected three times with 2×10^7 cells/mouse previously psoralen-photoinactivated, at the 14th, 21st and 28th day after the leukaemia transplant. Fig. 2 shows the observed mortality due to the leukaemia development; in the animal group without any treatment, the mortality increases very quickly and by the 20th day it is as high as 96%. In the group of animals treated with cyclophosphamide alone the mortality rises quickly until the end of the second week, then slows down, and finally stops to at 64%. The mean survival times observed in these two animal groups were practically equal (about 12 days). In the group of animals submitted to the combined therapy of cyclophosphamide and photoinactivated cells the mortality development is evident only during the first week; after this time only very few mice die and these after a long time. While the mean survival time shows only a little increase (18.5 days) the mortality is still very low, only 26%. Performing the χ^2 test, a very significant reduction in mortality resulted, even in comparison with the group of animals treated with cyclophosphamide.

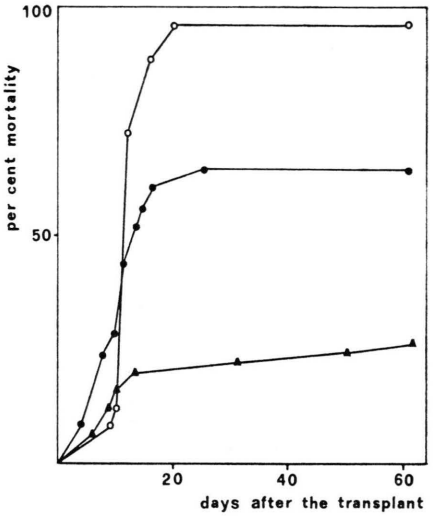

Fig. 2. L 1210 leukaemia combined therapy by cyclophosphamide and psoralen photoinactivated cells. Untreated animals (controls) –○–○–, animals injected with cyclophosphamide –●–●–, animals submitted to the combined therapy –▲–▲–.

Discussion

Psoralen photosensitization affects strongly DNA and RNA syntheses in L 1210 leukaemia cells; this result, similar to those obtained with other normal or tumor cells [14], is due to the psoralen photobinding to the pyrimidine bases of DNA, that loses its template activity.

Protein synthesis is also affected, but its inhibition requires higher radiation doses, as already observed in the other cell lines. This effect, certainly due to a psoralen photochemical interaction, does not appear to be connected with the photobinding to DNA. In fact, protein synthesis begin to decrease significantly at radiation doses that practically stop RNA synthesis.

On the other hand, psoralen photosensitization yielded a significant inhibition of protein synthesis even in absence of RNA synthesis, *i. e.* by incubating in the presence of actinomycin D. In these experiments, [^{14}C]amino acid incorporation was directed by pre-existing and not yet degraded RNA molecules. These experiments are consistent with the idea of direct damage to ribosomes due to the photobinding to RNA, to which psoralen can photoreact easily, as already observed [15].

The experiments of combined therapy showed clearly that the antigenic properties of L 1210 cells cannot be destroyed by psoralen photosensitization. It was necessary also to perform a chemotherapy treatment to reduce the number of the viable leukaemic cells to permit the immunological effect to become evident. In fact the simple therapy with the photoinactivated cells was without effect (data not shown here).

The combined therapy cyclophosphamide-psoralen photoinactivated cell strongly reduced the leukaemia development, yielding a result very significant statistically.

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