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Effect of Polycation Complexation on Methotrexate-Liposome Cytotoxicity

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ABSTRACT

Methotrexate and methotrexate-DEAE dextran complex were microencapsulated in positively charged liposomes. Their cytotoxicity was determined and compared with the cytotoxicity of control systems against L1210 mouse leukemia cells at 37° C in acetate buffer of pH 7.40 ± 0.05. The control systems used were acetate buffer, blank liposome, DEAE-dextran liposome and methotrexate solution. The methotrexate and methotrexate-DEAE dextran liposomes were lyophilized and the influence of lyophilization on their cytotoxicity was also examined. The methotrexate-DEAE dextran liposomes resulted in slightly higher mean growth ratios than the free methotrexate liposomes in

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nonlyophilized as well as lyophilized systems. The ED_{50} values for methotrexate and methotrexate-DEAE dextran liposomes were similar to that for the free methotrexate solution. This observation indicated that microencapsulation of methotrexate in liposomes either as a free drug or a complex has no effect on the cytotoxicity of the drug. In addition, lyophilization of liposome products does not seem to change their effectiveness.

INTRODUCTION

One of the major problems of cancer chemotherapy with cytotoxic drugs is the indiscriminate action of the drug on both diseased and normal cells. Considerable effort has therefore been directed towards increasing target specificity. Among the several vehicles suggested for the selective delivery of cytotoxic drugs to malignant cells, liposomes have apparently received the most attention.

Methotrexate (MTX), which falls in the cytotoxic drug category, has been clinically used for over twenty years in the treatment of acute leukemia and a variety of other malignancies[1]. Liposomes have been utilized as vehicles for MTX in several investigations[2-5]. In a previous communication we reported that the efficiency of entrapment and consequent retention of MTX in liposomes was enhanced by encapsulating the MTX-DEAE dextran complex instead of the free MTX[6]. The object of this research is to examine the cytotoxicity of a MTX-DEAE dextran complex, encapsulated in L-α-dipalmitolyl phosphatidyl choline (DPPC),

against L1210 mouse leukemia cells in comparison with the cytotoxcity of plain MTX liposomes and MTX solution. The influence of lyophilization on the cytotoxicity of liposome products was examined also.

EXPERIMENTAL

Methotrexate, USP (M.W. 454.44, Lederle Laboratories), DEAE-dextran (M>W. 500,000, Pharmacia Fine Chemicals), L-α-dipalmitoyl phosphatidyl choline (synthetic, amorphous 98%, anhydrous M.W. 734.10, Sigma Chemical Company), cholesterol (Eastman Kodak Co.), and stearylamine (anhydrous, M.W. 269.50, Sigma Chemical Company) were used as received. Liposomes were prepared by the chloroform film method[7]. The details of the preparation and storage have been described earlier[6].

The Cytotoxicity Study Procedures

The drug systems used in the cytotoxicity study were MTX solution, MTX liposomes and MTX-DEAE dextran complex liposomes both non-lyophilized and reconstituted solid powder from the lyophilization process. The control systems were acetate buffer, blank liposomes and DEAE-dextran liposomes.

Fisher's medium (Gibco H-11) with 10% horse serum (KC Biologic) was used in cell culture studies. To one 5-liter package of Fisher's concentrate, 4,500 ml of triple distilled, 500 ml of horse serum and 5.62 gms $NaHCO_3$ were added. The medium was sterilized by filtration and stored in a refrigerator. Prior to use, 100 units per ml of penicillin and 100 μg per ml of

streptomycin were added to the medium.

Stock cultures of the L1210 cell line, which were obtained from EG & G Mason Research Institute, a supplier for the NCI, were grown in Blake bottles at 37°C. The medium was prewarmed to 37°C and the cultures were split on Monday and Friday the week of the experiment. One hundred fifty five milliliters of culture was initially inoculated with 6×10^4 cells per ml. After 3-4 days growth, a concentration of 0.8×10^6 cells per ml was attained.

Cells for the cytotoxicity assay (L1210 cells) were obtained from a spinner culture (cells in logarithmic growth phase). The spinner cultures were grown in screw-capped Erlenmeyer flasks stirred by a magnetic stirrer. An aliquot containing 3×10^5 cells per ml was added to the pre-warmed (37°C) Fisher's medium a day prior to the assay. The spinner culture size was 200 ml. After 24 hours, the cell concentration was 0.8×10^6 - 1.5×10^6 cells per ml.

Prewarmed 37°C medium containing 6.6×10^4 cells per ml was prepared minutes before cells were dispersed into individual growth tubes. The growth tubes contained 3 ml of cells and 1 ml diluted test sample. The final cell concentration was 5.1×10^4 cells per ml which was the number of cells per ml at the baseline in the cytotoxic experiment.

The samples were diluted in series with Fisher's medium containing 10% horse serum; each sample was diluted into five to

six dilution levels from 1:4 to 1:4 x 10^8. Two tubes were prepared at each dilution level. The control tubes, which were the tubes without any test samples but containing 3 ml of cells and 1 ml of medium, varied in numbers according to the formula 2 $\sqrt{n}$ where n was the number of test samples. The resulting experiment tubes were then stoppered with gum rubber stoppers and incubated at 37°C for 48 hours. The number of cells per ml in each tube was determined by a hemocytometer and a coulter counter. The ED_{50} values and the percent mean growth ratio were used to evaluate the results. Mean values were utilized in all of the calculations.

The percent mean growth ratio was calculated as

$$\% \text{ mean growth ratio } (\%Y) = \frac{\text{mean no. of cells/ml in the test tube-mean baseline}}{\text{mean no. of cells/ml in the control tube-mean baseline}} \times 100 \quad (1)$$

The baseline was the mean cell inoculum on initiation of control. The effective diluted concentration that inhibited growth by 50% of the growth (ED_{50}), was calculated from the slope (B) and intercept (A) of the linear relationship between % mean growth ratio (%Y) and log concentration (X). The calculated ED_{50} values can be obtained from the following equations:

$$\%Y = A + B\,X \quad (2)$$

$$50\% = A + B\ (\text{Log } ED_{50}) \tag{3}$$

$$ED_{50} = 10^{\frac{50-A}{B}}\ \text{mcg/ml} \tag{4}$$

RESULTS AND DISCUSSION

The percent mean growth values for the lyophilized and non-lyophilized form of the two liposome systems are listed in Table 1. At high concentrations, the percent mean growth ratios obtained from coulter counter were higher than those obtained from the hemocytometer. This is due to the fact that the coulter counter counts every single particle including both the dead cells and the live cells whereas the hemocytometer counts only the live cells. At low concentrations, however, this problem is not evident and, except for one case, there is good agreement between the two methods.

In the non-lyophilized liposome system, the MTX-DEAE dextran liposome system gave higher values of the percent mean growth ratio than the MTX-liposome system at similar concentration levels. This probably indicates that MTX was retained longer in the complexed liposome system than in the free-MTX-liposome.

For the lyophilized liposomes, differences in the percent mean grown ratio values are not easily seen. For example, at a concentration level of 2.5×10^{-5} mcg per ml of MTX, MTX-liposomes show a higher percent mean growth ratio than the MTX-

Table 1. Comparison of Percent Mean Growth Ratio of MTX Solution and Liposome Systems.

Non-lyophilized MTX-liposome			Non-lyophilized MTX-DEAE-dextran liposome		
Conc. ($\frac{mg/ml}{ml}$)	Coulter counter	Hemocytometer	Conc. ($\frac{mg/ml}{ml}$)	Coulter counter	Hemocytometer
1.9×10^{-3}	0	0	2.6×10^{-3}	0	0
1.9×10^{-4}	4.5	0	2.6×10^{-4}	26.8	0
1.9×10^{-5}	3.1	0.4	2.6×10^{-5}	6.5	2.1
1.9×10^{-6}	26.1	24.3	2.6×10^{-6}	81.6	106.7
1.9×10^{-7}	99.4	98.4	2.6×10^{-7}	99.3	100.6
1.9×10^{-8}	94.5	93.2	2.6×10^{-8}	91.0	81.8

Lyophilized MTX-liposome			Lyophilized MTX-DEAE-dextran liposome		
Conc. ($\frac{mg/ml}{ml}$)	Coulter counter	Hemocytometer	Conc. ($\frac{mg/ml}{ml}$)	Coulter counter	Hemocytometer
2.5×10^{-3}	0	0	2.5×10^{-3}	0	0
2.5×10^{-4}	27.1	0	2.5×10^{-4}	24.9	0
2.5×10^{-5}	6.0	0	2.5×10^{-5}	3.5	0
2.5×10^{-6}	17.1	15.2	2.5×10^{-6}	58.6	54.4
2.5×10^{-7}	91.6	68.8	2.5×10^{-7}	97.0	-
2.5×10^{-8}	82.0	78.4	2.5×10^{-8}	92.8	-
2.5×10^{-9}	96.3	101.9	2.5×10^{-9}	90.9	-

MTX Solution		
Conc. ($\frac{mg/ml}{ml}$)	Coulter counter	Hemocytometer
2.5×10^{-3}	0	0
2.5×10^{-4}	1.2	0
2.5×10^{-5}	2.1	0
2.5×10^{-6}	75.2	53.1
2.5×10^{-7}	85.7	73.2
2.5×10^{-8}	91.1	80.6
2.5×10^{-9}	84.4	83.6
2.5×10^{-10}	85.7	93.2

DEAE dextran liposome while the reverse is true at a MTX concentration of 2.5×10^{-6} mcg per ml.

The ED_{50} values obtained from the coulter counter and the hemocytometer are shown in Table 2. Within the limits of experimental error, the ED_{50} values obtained from the two instruments were similar and for this reason an average of the two values is also shown in Table 2. The control systems, which

Table 2. Comparison of ED_{50} from Coulter Counter and Hemocytometer.

System	Coulter counter	Hemocytometer	Average values
Acetate buffer	0.11 mmoles/ml	0.42 mmoles/ml	0.27 mmoles/ml
Blank liposome	0.07 mg PL/ml	0.03 mg PL/ml	0.05 mg PL/ml
DEAE-dextran liposome	2.8×10^{-3} mg DEAE-dextran/ml	2.1×10^{-3} mg DEAE-dextran/ml	2.4×10^{-3} mg DEAE-dextran/ml
MTX solution	1.8×10^{-6} mg $\frac{MTX}{ml}$	0.6×10^{-6} mg $\frac{MTX}{ml}$	1.2×10^{-6} mg $\frac{MTX}{ml}$
MTX-lipo-pellet	1.7×10^{-6} mg $\frac{MTX}{ml}$	1.1×10^{-6} mg $\frac{MTX}{ml}$	1.4×10^{-6} mg $\frac{MTX}{ml}$
MTX-DEAE-dextran-lipo-pellet	6.2×10^{-6} mg $\frac{MTX}{ml}$	4.4×10^{-6} mg $\frac{MTX}{ml}$	5.3×10^{-6} mg $\frac{MTX}{ml}$
MTX-lipo-lyo.	1.4×10^{-6} mg $\frac{MTX}{ml}$	0.3×10^{-6} mg $\frac{MTX}{ml}$	0.8×10^{-6} mg $\frac{MTX}{ml}$
MTX-DEAE-dextran-lipo-lyo.	2.4×10^{-6} mg $\frac{MTX}{ml}$	3.5×10^{-6} mg $\frac{MTX}{ml}$	2.9×10^{-6} mg $\frac{MTX}{ml}$

include acetate buffer, blank liposomes and DEAE-dextran liposomes, gave an ED_{50} value about 1000 times higher than the drug systems which include MTX solution, MTX-liposomes, and MTX-DEAE dextran liposomes both in lyophilized and non-lyophilized form. Therefore, any effect observed in the drug systems was due to the drug itself. The MTX-liposome system and the MTX-DEAE dextran liposome system, both in the lyophilized and the non-lyophilized form, exhibited ED_{50} values very similar to that of the MTX solution; therefore, they were considered to have the same effectiveness as the free drug. The ED_{50} values, which range from 0.85×10^{-3} mcg per ml to 5.3×10^{-3} mcg per ml, are considered to be similar since a specific ED_{50} value cannot be obtained for a single test system. For example, the positive control compound, NSC 9544, gives an ED_{50} value in the range of 1.7 mcg per ml to 7.7 mcg per ml[8]. Moreover, a higher

variability of the cell behavior in cell growth always occurs during the experiment[9-11].

The cytotoxicity studies indicated that the lyophilization process did not appear to change the effectiveness of the drug against L1210 cells. Perhaps of greater significance was the observation that when MTX was complexed with DEAE-dextran polymer and entrapped in the liposome system, the drug still had the same effectiveness as the free drug.

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