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Study on the Active Delivery of Methotrexate Microspheres with Mouse Monoclonal IgG in Tumour–Induced Mice

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To investigate the therapeutic applications of immuno microspheres containing cytotoxic agent in affinity-chemotherapy, Methotrexate-loaded microspheres of size ranging from 1 to 3 µm were prepared by emulsion cross-linking method. The drug-incorporated microspheres were conjugated with mouse monoclonal antibodies. *In vitro* cytotoxicity studies using Hep-2 cells were done and it was found that the Methotrexate immuno microspheres had more affinity towards recognizing and binding to the Hep-2 cells when compared to methotrexate microspheres. *In vivo* studies were done using mice and the targeting efficiency of the formulations to lungs, liver and kidney was studied. The efficacy of the formulations for their anti-tumour activity was also studied against the Dalton ascites lymphoma cell-induced tumour in mice. Various parameters like weight gain, percentage tumour weight inhibition, and packed cell weight were studied. Our results showed that the methotrexate immuno microspheres group was more therapeutically efficient in terms of better affinity and binding capacity than methotrexate microspheres and free methotrexate in protecting the mice from carcinoma.

Drug carrier systems have been recently investigated and introduced since many drugs are not able to reach at the target areas in the body at effective concentrations. Due to lack of site-specific drug delivery, the toxic effects of cytotoxic drugs on normal tissues generally hinder the efficiency of conventional chemotherapy in the treatment of cancer patients[1]. A more suitable way of circumventing this problem is to apply the drug carrier system[2]. During recent years, the use of microspheres as controlled-release targeting agents for anticancer drugs has received wide attention. Stabilization of albumin matrix in the manufacture of microspheres can be accomplished by cross-linking through glutaraldehyde using emulsified albumin solution in w/o emulsion[3]. Microspheres produced by chemical-crosslinking with glutaraldehyde possess a hydrophilic surface to which conjugation of monoclonal antibodies (mAb) could be achieved with relative ease[4]. Research in mAb-directed therapy has revealed problems such as partial displacement of mAbs, opsonization, lack of recognition of antigens *in vivo*, and low antigen densities, as a result, antibody-drug conjugates often do not bind efficiently with the target antigens[5]. Hence, the delivery probability of therapeutically sufficient amounts of drug to target cells with some antibody-drug conjugates is generally low. Therefore, systems such as immuno microspheres and immuno liposomes have been investigated to increase the probability of efficacy[6]. Hence, in our present work, we have studied the therapeutic application and carrier-mediated specificity of immuno microspheres through anti-cancer agents, in site-specific drug delivery system for cancer therapy.

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MATERIALS AND METHODS

Bovine serum albumin was obtained from Sigma, (St. Louis, MO.), mouse monoclonal antibodies from Genei Pvt, limited Bangalore, MEM medium was processed from Hi-Media, Mumbai, Dalton ascites lymphoma cells were processed from the Amala Cancer Centre, Thrissur, Kerala and other chemicals used were of analytical grade.

Preparation of microspheres:

Albumin microspheres were prepared by emulsion cross-linking technique in which glutaraldehyde was used as the cross-linking agent[6]. Two millilitre aliquot of 25% (w/v) albumin containing 30 mg of methotrexate were shaken well and allowed to stand for about 15 min. This was stirred at about 300 rpm. Glutaraldehyde saturated toluene was added drop wise. The dispersion was stirred at appropriate speed (300 rpm) for about 4 h. The microspheres were collected and washed in acetone and toluene with centrifugation. The amount of drug incorporated into albumin microspheres was estimated using UV-spectroscopy. Four other batches of microspheres were prepared by the above-mentioned method with varying amounts of drug (10, 20, 40 and 50 mg), respectively, for batches I, II, IV and V.

Preparation of immunomicrospheres:

In the preparation of methotrexate-loaded immunomicrospheres[7], 100 mg of methotrexate microspheres were incubated with 10 mg of mouse mAb dissolved in 10 ml of Hanks balanced salt solution (HBSS) for 30 min at 4°, then washed with cold HBBS to isolate the unconjugated monoclonal antibodies, and stored at 4°. Fig. 1 shows the immunomicrospheres.

***In vitro* cytotoxicity study:**

A comparative *in vitro* cytotoxic efficacy of free methotrexate, methotrexate Microspheres, methotrexate immuno microspheres, was investigated on Hep-2 cell-lines[8] where aliquots (2 ml) of HEp-2 ($1x10^5$)/ml were added to RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum and seeded into 12 well microtitre plates respectively followed by varying amounts of methotrexate (MTX) (50, 100 and 150 µg of methotrexate/well) were added and incubated at 37° under 5% atmospheric CO_2. The viable count after 3 days was determined with a haemocytometer or by trypan blue dye extrusion method.

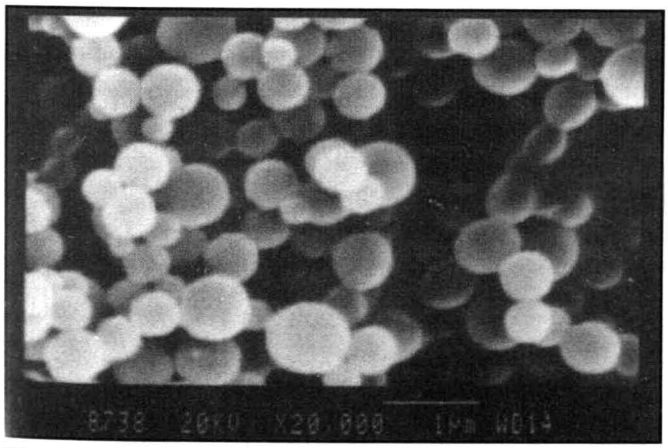

Fig 1: Scanning electron micrograph of methotrexate-loaded immunomicrospheres.

Bio-distribution study:

To compare the systemic effects of MTX-IMS with that of MTX-MS and MTX, a bio-distribution study[9] was carried out using a mouse model, in which, 32 healthy mice (25-30 g) were obtained and divided into four groups each containing 8 mice. Group I served as solvent control. Group II received free drug equivalent to 2.5-mg/kg-body weight. Group III received methotrexate incorporated Microspheres equivalent to 2.5mg/kg body weight and Group IV received immunomicrospheres equivalent to 2.5 mg/kg body weight. These experimental protocols have been approved by the Institutional Animals Ethics Committee.

After 12 h the mice were sacrificed and the lungs, the liver and the kidney were isolated and homogenized with 10 ml of sodium hydroxide (2%w/v). The homogenized organs were centrifuged at 12000 rpm for 30 min, and the supernatant was collected. Homogenized sample (1 ml) was loaded into the Millipore cartridges was washed with phosphate buffer and the retained methotrexate on the cartridge was then eluted using acetonitrile: 50 mM phosphate buffer (pH 3) (1:2). The eluted sample was analyzed by plotting the calibration curve using the peak areas of the standard

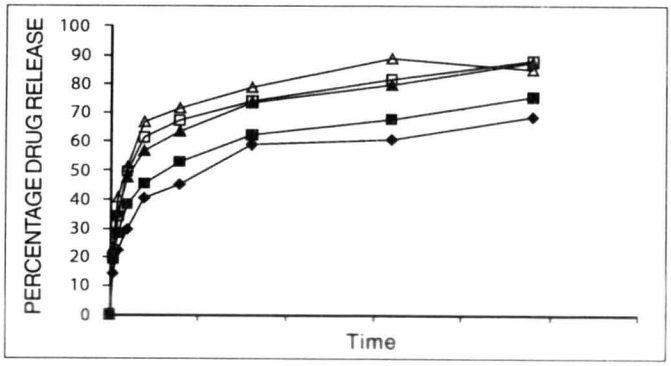

Fig 2: Dissolution profile of microspheres prepared with various drug to albumin ratios

Microspheres prepared with 10 mg of MTX (—◆—), 20 mg of MTX (—■—), 30 mg of MTX (—▲—), 40 mg of MTX (—□—) and 50 mg of MTX (—△—) were incubated in phosphate buffer (37°). Samples withdrawn at different time intervals and amount of MTX were determined by HPLC.

TABLE 1: TOTAL NUMBER OF VIABLE CELLS IN *IN VITRO* CYTOTOXICITY TEST

Group (µg/well)	Total viable cells present ($x10^4$)
Control	264.5±3.2
MTX-50	4.5±2.1
MTX-100	1.0±0.9
MTX-150	0
MTX-MS-50	74.5±3.8
MTX-MS-100	37.5±1.4
MTX-MS-150	31.0±2.8
MTX-IMS-50	22.5±3.2
MTX-IMS-1100	15.0±1.8
MTX-IMS-150	5.5±2.2

MTX; free methotrexate, MTX-MS; methotrexate-loaded microspheres, MTX-IMS; methotrexate-loaded immunomicrospheres. Each value represents the mean (n=3)

solutions against the concentration in ng/ml. The peak of the sample chromatogram was compared and the amount of methotrexate was calculated.

Study on antitumour efficacy:

To compare and evaluate the degree of specificity of drug-loaded immuno microspheres with that of free MTX and MTX-MS, tumour-binding capacity against Dalton ascites lymphoma cells was investigated. The anti-tumour efficacy was determined in terms of percentage tumour inhibition and packed cell weight. The study was carried out in 2 batches. In batch I, the mice were divided into 5 groups each containing 6 mice. The mice were induced with cancer with Dalton ascites lymphoma cells and the mice were allowed to grow with tumour. The drug treatment was started from the 2 d onwards. The drug was dispersed in Hanks buffer and administered i.v. through tail vein[9,10]. The experimental protocol is given below.

Mice of group I were not treated with drug and allowed to grow with cancer. This group served as control, mice of group II were received the solvent Hanks buffer and served as solvent control, mice of group III were received methotrexate equivalent to 2.5 mg/kg, mice of group IV were received methotrexate Microspheres equivalent to 2.5 mg/kg, and mice of group V were received methotrexate immuno microspheres equivalent to 2.5 mg/kg.

In Batch II, the mice were divided into groups as that of Batch I, but the treatment was started from the day11. During the period of study both the batches were evaluated for parameters like packed cell weight and hematological changes.

RESULTS AND DISCUSSION

All the five batches with different drug to albumin ratio were subjected to *in vitro* release study in a shaker cum incubator. The gradual release was observed for 24 h. It was found that the batch with 30 mg of drug showed a relatively better release profile with a cumulative percentage release of 87.3% and it was selected for conjugation with monoclonal antibodies. Fig. 2 shows the *in vitro* drug release from various batches.

In our attempt to investigate the affinity of free drug, MTX-MS and MTX-IMS on the immortal Hep-2 cell line *in vitro* after 3 d from the addition of drug, the total number of viable cells were counted by trypan blue dye extrusion technique. It was found that the number of viable cells was lower in cells treated with MTX-IMS than that of MTX Microspheres. The results are shown in Table 1. It has also been observed that MTX-MS was showing higher number of viable cells than free drug probably due to the slow diffusion of drug from the microspheres.

From the comparative bio-distribution studies of MTX-

TABLE 2: THE AMOUNT OF METHOTREXATE DISTRIBUTION TO VARIOUS ORGANS

Organ	MTX (ng/organ)	MTX-MS (ng/organ)	MTX-IMS (ng/organ)
Kidney	156.8±0.8	937.3±0.6	203.3±1.7
Liver	204.8±1.2	235.0±1.4	979.5±2.2
Lungs	234.9±1.4	399.6±1.2	256.6±2.4

MTX; free methotrexate, MTX-MS; methotrexate-loaded microspheres, MTX-IMS; methotrexate-loaded immunomicrospheres. Each value represents the mean (n=3)

TABLE 3: THE PACKED CELL WEIGHT AND % TUMOUR INHIBITION IN BATCHES OF ANIMALS WITH DLA TUMOR.

Group	Packed cell weight (g/mice)	% Tumor weight inhibition (test/control x100)
Control. Batch (I)	8.2	0.0
Solvent Control	8.0	2.4
MTX	6.1	25.1
MTX-MS	3.1	62.8**
MTX-IMS	2.0	75.2**
Control. Batch (II)	10.3	0.0
MTX	8.2	20.4
MTX-MS	7.0	32.3**
MTX-IMS	6.5	37.0**

**Significantly different at $p<0.01$. MTX; free methotrexate, MTX-MS; methotrexate microspheres, MTX-IMS; methotrexate immunomicrospheres. Each value represents the mean (n=3)

IMS, MTX-MS and free MTX, it was observed that the immuno microspheres were also effectively captured by the organs of RES, with predominant localization of drug in liver. The amount of drug distributed to various organs from MTX-IMS, MTX-MS and free MTX is tabulated in Table 2. From the table it is clear that the drug distribution from MTX-IMS was relatively higher in the liver, lungs and the kidney than from MTX-MS and free MTX. This indicates that the systemic clearance of immuno conjugates by RES to a greater extent than that of MTX-MS and free MTX, however MTX-MS showed a relatively higher amount of drug distribution to lungs than the free MTX and MTX-IMS, probably due to the passive uptake of MTX-MS by the lung capillaries.

The packed cell weight was estimated in both the batches of mice, as it is shown in Table 3. Through the packed cell weight, the percentage tumor weight inhibition of three groups such as MTX-free, MTX-MS, and MTX-IMS was compared with that of control. To compare the efficacy of MTX-IMS system over the other two systems in preventing the tumour weight, the test of significance was carried out. It was found that, the percentage tumour weight inhibition was significantly higher in both the batches of animals treated with MTX-IMS than the other groups and control ($P<0.01$) but to evaluate the antitumour efficacy of these systems more critically, an elaborative study through a variety of different tumour models are still required. Such studies may be helpful to understand the mechanistic rationale for their enhanced protection against variety of tumors.

The above findings demonstrated that the MTX-IMS group was more effective in terms of better cell affinity and binding capacity than the MTX-MS and free MTX and control groups, against the selected cancer cells. But to exploit its therapeutic role in cancer chemotherapy, still extensive investigations against human cancer cells are required. It would be worthy to investigate the comparative efficacy of these systems, against various routes of administration, to understand their relative therapeutic enhancement and increased vascular permeability in the treatment of cancer.

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