Application of radioimmunoassay to monitor treatment of human cerebral gliomas with bleomycin entrapped within liposomes

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SUMMARY A radioimmunoassay was assessed for its suitability for monitoring the blood and urinary concentrations of bleomycin entrapped within large unilamellar liposomes in patients receiving therapeutic doses of the drug. Bleomycin entrapped within liposomes was administered by direct intracerebral injection to three patients with grade III to IV cerebral gliomas, twice weekly for up to six weeks. No clinically important toxic effects were observed which could have been attributed to these preparations, although all three patients showed progressive deterioration in their clinical condition. This type of treatment may be more beneficial to patients at an earlier stage of their disease.

Cerebral gliomas are the most common form of primary brain tumour; they occur at an average age of only 45 to 50 years, yet have an extremely poor prognosis.¹ Astrocytomas account for about 75% of all cerebral gliomas, and about three quarters of these are grade III to IV tumours, which are rapidly growing and locally infiltrative and hence impossible to eradicate completely by surgery. Surgery, radiotherapy, and more recently chemotherapy and immunotherapy have improved the outcome to some extent, but nevertheless, the condition is still almost uniformly fatal, with poorly differentiated gliomas having a reported median survival time of five to 13 months.² In view of the large kinetic difference between cell division of the tumour and surrounding normal brain tissue, chemotherapy might be expected to be an effective treatment, but it is difficult to achieve a sustained therapeutic concentration of the drug at the tumour site after systemic administration.

Local injections of antimitotic drugs into cerebral tumours has been attempted, but owing to the vascularity of the tumour bed it has not been possible to maintain therapeutic concentrations of the drug at the tumour site for any length of time.³ This suggests that an inert depot preparation of the drug injected locally into the tumour mass to produce a sustained release of agent over a greater length of time may be a more

effective approach while minimising the side effects that would occur from high dose systemic administration.

Liposomes have been extensively studied recently as a possible vehicle for giving chemotherapy in an attempt to target drug activity specifically to diseased areas of the body.⁴ Liposomes are phospholipid bilayers formed into spheres in the presence of water and they can be made to incorporate a wide variety of agents including drugs. Many different phospholipids have been incorporated into liposomes which may also contain varying amounts of cholesterol, together with other lipids that confer a net overall charge on the liposome membrane (such as phosphatidic acid, acetyl phosphate, stearylamine). The preparation of liposomes is relatively straightforward, and the techniques available have been adequately reviewed elsewhere.⁵ The two most commonly used techniques entail the sonication of an aqueous dispersion of phospholipids to produce small unilamellar vesicles,⁶ or alternatively, the sonication of an aqueous solution of the drug together with an organic solution of lipids to produce a reverse phase emulsion, followed by rotary evaporation of the organic solvent to yield large unilamellar vesicles.⁷

When administered intravenously to animals or to human beings, liposomes accumulate predominantly in the liver and spleen, with only minimal selective uptake into malignant tissue,⁸ and this has recently been shown to be due to the inability of all but the very

smallest of liposomes to penetrate the continuous capillaries of liver, spleen, lung or other tissues.⁹ The difficulties might be overcome by local injection directly into the tumour mass of liposomes containing entrapped antimitotic drugs.

Adams *et al* showed that liposomes of defined composition may be safely used as carriers of therapeutic agents into the mouse central nervous system.¹⁰ We determined the optimal conditions for the incorporation of high concentrations of two antitumour drugs (bleomycin and vincristine) into negatively charged liposomes.¹¹ A delayed release of these drugs from the liposomes was shown *in vitro* and dose response curves were similar for the two drugs whether given as the free or liposome entrapped drug to a human glioma cell line (U251MG). Studies on the intracerebral injection of bleomycin, free and entrapped, within liposomes in rats have shown no detectable cerebral toxicity but a delayed clearance from the injection site when given entrapped within liposomes.¹²

We subsequently confirmed that sterile, negatively charged liposomes of defined composition can safely be administered postoperatively to patients with grade IV cerebral astrocytomas, through Ommaya reservoirs, direct to the residual tumour bed without noticeable side effects.¹³ Furthermore, when bleomycin entrapped within liposomes is injected into the tumour bed the maximal serum concentrations achieved are about one tenth of those obtained after injections of similar concentrations of free drug, and the 24 hour urinary clearance is considerably reduced.

These results suggest that bleomycin given intracerebrally at high concentration entrapped within liposomes, to patients with cerebral gliomas, should act as a slow release depot, enabling high infrequent doses of the drug to be given over a protracted period. We report on the use of a bleomycin radioimmunoassay suitable for the estimation of the drug in blood, urine, and tissue components in patients receiving liposome treatment, in addition to preliminary studies of the application of this novel form of treatment to patients with grade IV cerebral gliomas.

Material and methods

Dipalmitoyl-DL-phosphatidylcholine (DPPC), cholesterol, dipalmitoyl-DL-phosphatidic acid (PTA), and thimerosal were obtained from Sigma Chemicals, Poole, Dorset. ¹²⁵I-sodium iodide was purchased from Amersham International, Buckinghamshire. Iodogen (1,3,4,6-tetrachloro-3,6-diphenylglycouril) was obtained from Pierce Chemical Company, Cambridge. Gelatin was purchased from Oxoid Ltd, Basingstoke, Hampshire.

Bleomycin antisera (batch G/S/B16-IIAC) and don-

key antiserum (batch HPD.43-IIC) were provided by Guildhay, University of Surrey. Bleomycin was kindly donated by Lundbeck Ltd, Luton, Bedfordshire. Polyethylene glycol 6000, methanol, chloroform, and other commonly used laboratory reagents were purchased from BDH Chemicals, Poole, Dorset.

Radioiodination was performed according to the Guildhay procedure with minor modifications.¹⁴ Bleomycin (5 µg) in 10 µl of 0.2M phosphate buffer (pH 7.4) was added to the bottom of a polypropylene phial that had been coated with 20 µg iodogen. ¹²⁵I-sodium iodide (5 µl) (0.5 mCi) was added and the reaction was allowed to proceed for eight minutes at room temperature, after which 0.2 ml of 0.2M phosphate buffer (pH 7.4) was added. Separation of free iodide from ¹²⁵I-labelled bleomycin was achieved using a Bio-Rad 10 cm × 0.7 cm column containing 1 g/l gelatin and 170 mg/l thimerosal, with elution by the same buffer.

RADIOIMMUNOASSAY

The radioimmunoassay was carried out using a polyethylene glycol-double antibody phase separation technique according to the following protocol. Standards or samples (50 µl) were added to 200 µl of ¹²⁵I bleomycin (24 000 cpm) in phosphate buffer (0.05M, pH 7.4) containing 1 g/l gelatin and 170 mg/l thimerosal, followed by 200 µl of bleomycin antiserum in the same buffer to give a final antiserum dilution of 1 in 7200. After overnight incubation at room temperature 200 µl of donkey antiserum diluted 1 in 40 in the same buffer was added followed by 200 µl of 120 g/l polyethylene glycol 6000. After 30 minutes' incubation at room temperature all tubes were centrifuged for 10 minutes at 2000 g, the supernatants removed by evacuation, and radioactivity of the pellets counted for 30 seconds on a Wilj System 2000 gamma counter.

PRODUCTION OF LIPOSOMES

Liposomes were prepared by the reverse phase technique⁷ under conditions appropriate for maximal incorporation of bleomycin within the aqueous internal space of the liposomes. Bleomycin (15 mg) in 1 ml of sterile 0.02M phosphate buffer (pH 5.8) was sonicated with 66 µmol of DPPC:cholesterol:PTA (7:2:1) in 2 ml of diethyl ether:chloroform (1:1) for two minutes at an amplitude of 10 µ on a Fison's Soniprep 150 ultrasonic homogeniser. Organic solvent was removed by rotary evaporation of the reverse phase emulsion at 45°C to leave a viscous gel that subsequently formed an aqueous suspension on prolonged evaporation. Separation of liposome entrapped bleomycin from free bleomycin was carried out with a Sepharose-6B column under aseptic conditions, with

collection of the opaque liposome fraction directly into sterile evacuated glass 5 ml phials.

The effect of intact liposomes in serum on the radioimmunoassay of bleomycin was assessed by carrying out the assay in the presence of various dilutions of liposomes, free or containing entrapped bleomycin in normal human serum. The percentage binding of label to antiserum under these conditions was compared against a standard curve produced by diluting known amounts of pure bleomycin in normal pooled human serum.

The effects of various liposome disrupting agents on the liposomes and on the subsequent radioimmunoassay were assessed by treating serum containing free liposomes or liposome entrapped bleomycin, with methanol, chloroform, or 0.5% Tween 80.

Studies on patients were carried out at the Atkinson Morleys Hospital and Hurstwood Park Neurological Centre after local ethical committee approval. After clearance studies in eight patients with cerebral glioma¹³ a further three patients were selected for an attempted trial of chemotherapy. In all three, cerebral tumour was diagnosed clinically and confirmed by computed tomography scanning. Histological examination showed grade III or IV cerebral glioma after surgery for partial removal of the tumour and placement of an Ommaya reservoir (table 1).

The patients were given several days to recover from the surgical procedure after which 0.5, 1, 2, or 6 mg of free bleomycin was injected into the tumour bed via the Ommaya reservoir. A 5 ml blood sample was obtained from each patient at 0, 0.5, one, two, four, and six hours after the injection together with a 24 hour urine sample. Three to four days after injection of free drug a trial of bleomycin entrapped within liposomes was started with the intention that this should be given twice a week for six weeks. The same quantity of bleomycin was administered entrapped within liposomes as was given in the free form to each

Table 1 *Tumour site and histological diagnosis in three patients receiving intracerebral treatment with bleomycin entrapped within liposomes*

Case No	Sex	Age (year)	Site and size of tumour	Histological diagnosis
1	M	73	Large, right temporo-parietal lesion	Grade IV astrocytoma
2	F	66	Large lesion in the right frontal region extending to the surface of the brain	Grade III astrocytoma
3	F	16	Large, well localised tumour in right temporal region	Grade III to IV astrocytoma

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patient, and blood specimens and whenever possible, urine specimens, were obtained as outlined above. The patients' clinical conditions and response to the injections were continuously monitored and computed tomography scans were repeated at three weekly intervals whenever possible.

Results

RADIOIODINATION

Iodination using the iodogen technique produced a high specific activity label which consistently gave an antibody binding of 85% or greater in the presence of excess bleomycin antisera. The label was stable for up to eight weeks with only a slight reduction in maximal antibody binding over this period.

RADIOIMMUNOASSAY

The radioimmunoassay using a double antibody-polyethylene glycol phase separation was shown to have an interassay coefficient of variation of 3.5% and an intra-assay coefficient of variation of 7.5% at a concentration of 50 µg/l and a detection limit of 40 pg per tube.¹⁴ The technique, like many immunoassays, was slightly susceptible to matrix effects, and consequently it was necessary to use a standard curve consisting of bleomycin in normal pooled human serum, normal urine, or normal urine appropriately diluted, depending on the nature of the specimen being analysed.

PRODUCTION OF LIPOSOMES

Detailed studies of the factors affecting the incorporation of bleomycin within liposomes have been published elsewhere.¹¹ These studies have led to the production of liposomes with entrapment efficiencies in the region of 55 to 60% of total drug. Subsequent studies have shown that sterile non-pyrogenic preparations can be produced consistently, and furthermore, that by storage of these preparations at 4°C before use, 85% of the bleomycin remains entrapped within the liposomes after eight weeks' storage. Samples of all liposome preparations used in this study underwent microbiological examination and culture for up to seven days before being used and all preparations were given within three weeks of production.

EFFECT OF LIPOSOMES ON THE RADIOIMMUNOASSAY OF BLEOMYCIN

The presence of bleomycin free liposomes in serum had no effect on the binding of antiserum to bleomycin label or on the shape of the bleomycin standard curve, except at liposomal lipid concentrations of 100 µmol lipid/l of serum, when a 5% depression of binding of bleomycin label to antiserum was observed at zero bleomycin concentration (table 2). This concentration

Table 2 *Effect of bleomycin free liposomes in serum on binding of bleomycin antisera to label*

Normal lipid concentration in serum ($\mu\text{mol/l}$)	Percentage (SEM) binding of radiolabel by bleomycin antisera
0	45.9 (1.3)
0.63	43.6 (1.0)
1.25	45.1 (0.3)
2.5	45.1 (1.1)
5.0	43.9 (0.7)
10.0	46.3 (0.7)
100.0	41.5 (0.9)

is vastly in excess of the liposome concentration likely to be found in serum, even immediately after direct intravenous administration. Bleomycin when entrapped within liposomes was not detected by the immunoassay, however, and it was therefore necessary to release the drug from the liposomes before analysis. It has been reported that liposomes can be disrupted by several agents including chloroform, diethyl ether, and non-ionic detergents, but these studies also showed that the incorporation of Tween 80 into the assay buffer to a final concentration of 0.5% had no effect on the release of bleomycin from liposomes. Pretreatment of serum with an equal volume of methanol or chloroform resulted in complete release of bleomycin from liposomes, although samples pretreated with methanol depressed antibody binding of label by about 5% of total counts and samples treated with chloroform depressed binding by about 20% of total counts. When carrying out this procedure it was therefore necessary to pretreat serum standards in a similar manner before radioimmunoassay.

PATIENTS

The treatment schedules in the three patients together with peak serum bleomycin concentrations achieved and 24 hour urinary bleomycin excretion are shown in figs 1a-c.

All three patients seemed to tolerate these preparations well, the only apparent adverse effects observed

being mild transient headaches and prolonged drowsiness in case 1, particularly after the administration of the higher (6 mg) dose of bleomycin, and some nausea and slight drowsiness in case 2. These mild effects were not better or worse towards the end of the treatment schedule in either patient.

After injection of free drug in each patient the bleomycin rapidly appeared in the blood, although the time at which peak blood concentrations occurred varied with each patient. Peak blood concentrations following administration of similar amounts of bleomycin entrapped within liposomes were appreciably lower and were sufficiently near to the detection limit of the assay in most cases that it was not possible to determine accurately the serum half life. Similarly, the 24 hour urinary excretion of bleomycin was also lower although the extent of the reduction varied with each patient and with the dose given (table 3). Following direct radioimmunoassay of the specimens the analyses were repeated after pretreatment with both methanol and chloroform in all cases. In no case was there any pronounced change in the results obtained with this treatment.

In view of the rapidly deteriorating state of all three patients it was difficult to determine whether there was any therapeutic benefit. Case 1, a 73 year old man and case 2, a 66 year old woman, both showed some slight improvement in their clinical condition after the initial administration of liposomes, but this was short lived and both were eventually lost to the study because of the progressive deterioration in their condition. Case 3, a 16 year old girl, received a course of nine injections of liposomes over six weeks. Further computed tomography scans showed evidence of a second lesion in the same cerebral hemisphere in the region of the anterior corpus callosum. This was increasing in size at a rate greater than the original lesion, which might suggest that a beneficial therapeutic effect was being witnessed on the original tumour. She was discharged home and died three weeks later. A post mortem examination was not performed.

Table 3 *Blood and urinary bleomycin concentrations after intracerebral injection of bleomycin, free or entrapped, within liposomes*

Case No	Bleomycin (mg)	Peak serum bleomycin concentration ($\mu\text{g/ml}$)		24 Hour urinary bleomycin (% of administered dose)	
		Free	Entrapped	Free	Entrapped
1	2	22.5	0.8	46	1.9
	6	40.5	9.4	22	3.9
2	1	9.2			
	0.6		2.2* (2.0-2.6) 4.0† (1.8-6.6)		
3	0.5	10.8		53	23.6‡ (17-30)

*mean of three studies; †mean of nine studies; ‡mean of six studies.

Fig (a-c) Administration of bleomycin entrapped within liposomes in three patients with grade III to IV cerebral gliomas; (a) case 1; (b) case 2; (c) case 3. (24 hour urinary bleomycin excretion (as percentage of total dose) is given in parentheses).

Discussion

Although liposomes have been extensively studied during the past 10 to 15 years with a view to their application as a means of selective delivery in man, the study described in this paper is one of only a handful to have reported their use in the attempted treatment of disease. The preparations used here were simply acting as a non-toxic biodegradable slow release source of the chemotherapeutic agent, although there is some evidence that liposomes may be adsorbed on to tumour cells, thus leading to a reduction in the diffusion distances.

The availability of antisera specific for bleomycin has enabled us to monitor closely the appearance of the drug in blood and urine following the direct intratumoural administration of these preparations and gives indirect evidence that the drug remains at the tumour site for much longer when administered within liposomes. Bleomycin radioimmunoassays carried out after pretreatment of the specimen with methanol or chloroform showed that there was no evidence of leakage of intact liposomes into the bloodstream via the blood-brain barrier impaired by tumour. These indirect studies need to be confirmed by more direct techniques, and a preliminary study has been carried out using direct gamma scanning of the tumour bed following the administration of ¹³¹I-iodide labelled bleomycin entrapped within liposomes (unpublished observations). This study has shown considerably increased retention of the ³¹I-iodide at the tumour site 24 and 48 hours after administration of liposome entrapped drug compared with injection of free drug, thus adding further weight to the conclusions derived from this and previous studies.

No definite evidence of clinical response to treatment was noted in this small trial, but this was not unexpected. All three patients had presented at a late stage in the progression of their disease, having already undergone surgery and radiotherapy, and none was able to tolerate a full six week course of treatment owing to complications unrelated to toxicity of the preparations. Having shown that this treatment is relatively non toxic, however, even after repeated twice weekly injections, and that there is a sustained release of the drug following its administration entrapped within liposomes, it is anticipated that this will encourage its use in patients with both grades III-IV gliomas at an earlier stage of their illness.

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