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THE MANUFACTURE AND TUMOUR CELL UPTAKE OF NANOPARTICLES LABELLED WITH FLUORESCEIN ISOTHIOCYANATE

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Nanoparticles with a diameter of between 100 and 800 nm can be made from gelatin or albumin using a desolvation and hardening technique. Fluorescein isothiocyanate (FITC) can be bound to the surface of such nanoparticles. The conjugated gelatin nanoparticles were phagocytozed by some experimental tumour lines.

A number of malignant cell lines are known to be phagocytic. Some cytotoxic agents have been incorporated into liposomes[1,2] and the product has been taken up by phagocytic cells. However liposomal products normally can only be stored for a maximum of a few weeks under the most stringent conditions. This type of therapy will be limited to those institutions which can make and use liposomes quickly.

Nanoparticles may be a commercially viable alternative colloidal drug delivery system. Nanoparticles are made by cross-linking proteins such as gelatin or albumin with glutaraldehyde under conditions when the protein is almost coacervating[3,4]. Discrete solid particles with a diameter of between 100 and 800 nm can be obtained in a freeze dried state. A detailed discussion of the nanoparticle concept is given elsewhere[5,6].

FITC will conjugate with proteins under alkaline conditions. Ward and Fothergill[7] consider the ε-amino group of lysine as the probable site of conjugation in discrete protein molecules. Such conjugates are used clinically in direct tracing, plasma clearance and immunological clearance procedures.

As a first step in the development of an economic cytotoxic delivery system, it has to be shown that nanoparticles can be taken up by tumour cells. This note describes the manufacture of nanoparticles, the tagging of their surface with FITC and the results of incubating tumour lines with the tagged nanoparticles.

EXPERIMENTAL

(i) Nanoparticle Manufacture. Gelatin nanoparticles were prepared using gelatin type II (lot no. 28B-0590) from swine skin (Sigma Chemical Company, USA), Human Serum Albumin, Cohn Fraction V (HSA) (Commonwealth Serum Labs., Melbourne), Polysorbate 20 BPC (ICI Australia Ltd), and glutaraldehyde 25 per cent w/v aqueous solution (Koch-Light Laboratories, England). Sephadex G-50m

(Pharmacia South Seas Pty. Ltd., Sydney) and Sodium sulphate anhydrous (Ajax) were used as received. All other chemicals used were AR grade.

A Nepho-colorimeter, Model 9, (Coleman Instruments Corporation, USA) was used to monitor changes in intensity of scattered light during the desolvation/resolvation steps in the nanoparticle manufacture. A Silverson laboratory homogenizer fitted with a microhead, an Isco Fraction Collector (Model 1200 Pup) and a Dynavac FD2 Freeze Drier were also used.

Ten ml of a solution of 1% gelatin II and 0.5% Polysorbate 20 was made and equilibrated at 35°C. Seven ml of 20% w/v sodium sulphate was added, whilst stirring with a magnetic stirring bead, until the intensity of scattered light, monitored by a nephelometer, rose rapidly and the system was permanently faintly turbid. 1.2 ml of isopropanol was added until the turbidity disappeared and the intensity of the scattered light decreased to a predetermined point (nephelos number of under 50).

The laboratory homogenizer was then used to agitate the system as 0.4 ml of a 25% aqueous solution of glutaraldehyde was added in one aliquot. The hardening process was allowed to continue for 11 minutes at 35°C when it was terminated by the addition of 5 ml of a 12% sodium metabisulphite solution. After sufficient time for the termination reaction to occur, the crude system was frozen in a dry ice/acetone bath and dried overnight on the freeze drier.

To make albumin nanoparticles, 10 ml of a 5% HSA and 2% Polysorbate 20 solution was desolvated at 35°C with 4.2 ml of 40% ammonium sulphate and resolvated with 0.5 ml of isopropanol. 0.2 ml of 5% glutaraldehyde was added in one aliquot and homogenized for 10 minutes. One ml of 12% sodium metabisulphate was then added to terminate the reaction. The crude system was then freeze dried.

Excess salts and low molecular weight species can be removed by passing 30 ml of a 10% crude nanoparticle system through a 320 ml Sephadex G-50 m column. The nanoparticles appear in the void volume when 0.04% chlorbutol solution is used as the eluant. The low molecular weight species are eluted after the nanoparticles. The appropriate fractions can then be collected and freeze dried. Scanning electron micrographs of the powdered desalted product were made.

Freeze dried nanoparticles have been stored for one year and then their dispersibility in aqueous systems evaluated. Five female mice were given weekly intravenous injections of drug free nanoparticles over a twelve week period. The mice were then held for a further twelve weeks. Gelatin nanoparticles were autoclaved at 121°C for 15 minutes.

(ii) Conjugation with FITC. The coupling method outlined by Nairn[8] was followed. 80 mg of purified nanoparticles were dissolved in 4 ml of water and 1 mg of FITC in a phosphate buffer of pH 9 added slowly. The pH was raised to 9.5 with 0.1 M Na_3PO_4 and the mixture allowed to react at 25°C for 30 minutes.

Salts and unreacted FITC were removed by gel chromatography through Sephadex G-50 in a cold room maintained at 4°C. The void volume fractions containing the conjugate were freeze dried The efficiency of binding was determined by collecting the fractions containing the unbound FITC, making up to 250 ml and measuring the absorbance at 492 nm on a Cary 118 UV-VIS spectrophotometer.

1 mg of FITC labelled gelatin nanoparticles were aseptically placed in 10 ml of tryptone soya bean broth and incubated at 37°C for 48 hours. No evaluation of viral contamination has been undertaken but such contamination could be avoided by selection of starting materials and control of manufacturing procedures.

(iii) Cellular Uptake. FITC labelled nanoparticles were incubated in a α modification - Eagles minimal essential medium for 18 hours with a number of experimental tumour lines. The tumour lines (all of which are transplantable into BALB/c mice) included EMT6 (mouse mammary); WEHI-3 (mouse myelomonocytic) and SP-1 (rat squamous cell). These cell lines are known not to take up free FITC. Samples of the incubated cells were examined in a fluorescent microscope. Cellular uptake of the nanoparticles was seen as a well defined yellow cell against a black background.

RESULTS AND DISCUSSION

(i) Nanoparticle Manufacture. The micrograph (Figure 1) shows spherical, fairly uniform sized gelatin nanoparticles of approximately 500 nm diameter. The albumin nanoparticles used were of similar size. The other similar nanoparticles which had been

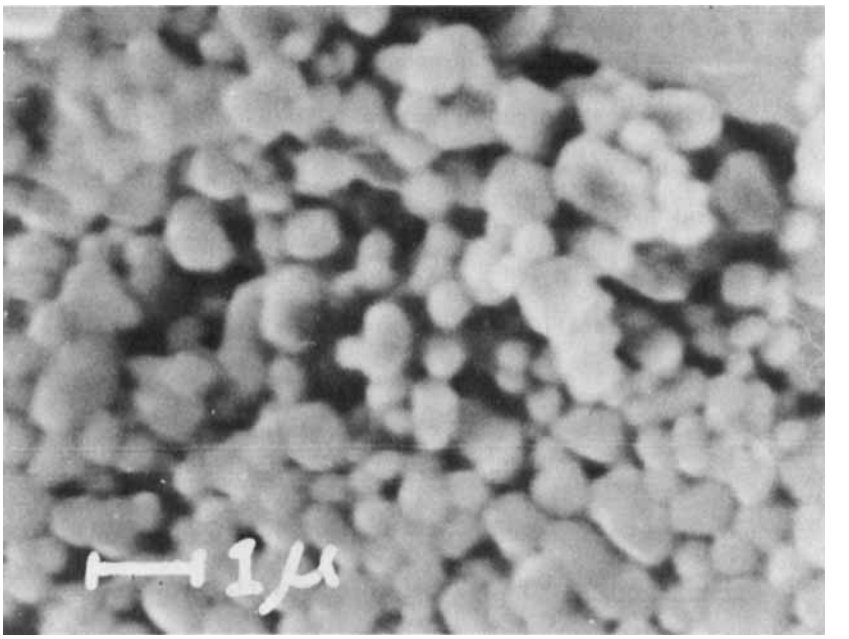

FIGURE 1 Scanning electron micrograph of gelatin nanoparticles after conjugation with FITC

stored for one year were successfully dispersed in aqueous systems. The extended weekly injections into mice produced no ill effects and the mice appeared normal after the further twelve week holding period.

(ii) Conjugate with FITC. Using the gelatin nanoparticles, the binding reaction was about 60% efficient. However, with the albumin nanoparticles it was about 85% efficient. Since FITC can be conjugated to the surface of gelatin or albumin nanoparticles, it shows that surface amino groups are not totally consumed in

the glutaraldehyde crosslinking process. These amino surface sites may be utilised to bind drug molecules and then the nanoparticle system used to deliver the drug to its desired site of action. This approach is currently under examination.

For parenteral use it is important that the preparation is sterile and apyrogenic. It is possible to obtain high quality gelatin for medical use and gelatin preparations can be sterilized by autoclaving without excessive degradation occurring. Prolonged heating of the solutions will hydrolyze the gelatin and produce smaller peptide chains[9]. No gross changes were observed in the scanning electron microscope when gelatin nanoparticle preparations were autoclaved. No turbidity developed when FITC labelled gelatin nanoparticles were incubated in the tryptone soya bean broth, indicating the absence of aerobic bacteria and fungi in the nanoparticle product.

(iii) Cellular Uptake. The mouse mammary tumour EMT 6 had up to 80% of the cells showing incorporation of the labelled gelatin nanoparticles. SP-1 had most of its cells showing uptake, but that uptake was not as strong as that with EMT 6. The mouse myelomonocytic tumour WEHI-3 had about 10% of the cells showing strong uptake. None of the cell lines tried took up albumin nanoparticles labelled with FITC. Other cell lines are currently being screened for both gelatin and albumin nanoparticle uptake.

Since some cell lines show uptake of nanoparticles, a new cytotoxic delivery system may be possible. However, before this can be realised, cytotoxic agents would have to be incorporated

into nanoparticles and those nanoparticles evaluated against the cell line screen. A suitable formulation procedure would have to be devised to direct the nanoparticles to the tumour cells rather than to the phagocytic Kupffer cells of the liver. Likewise as it is known that nanoparticles can be tagged with ^{99m}Tc[6] it may be possible to develop a tumour scanning agent.

CONCLUSIONS

Gelatin and albumin nanoparticles can be surface conjugated with fluorescein isothiocyanate. This shows that not all the surface amino groups are consumed in the manufacture of nanoparticles. Some cell lines take up gelatin nanoparticles. The feasibility of a cytotoxic delivery system based on nanoparticles now has to be explored.

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