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Adsorption of monoclonal antibodies to polyhexylcyanoacrylate nanoparticles and subsequent immunospecific binding to tumour cells in vitro

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Summary

The coating of polycyanoacrylate nanoparticles with a monoclonal antibody (anti-tumour osteogenic sarcoma) was studied in order to evaluate its potential for tumour targeting. It was shown that monoclonal antibody was well adsorbed onto the surface of the nanoparticles and that it retained its capacity to bind specifically to target tumour cells in vitro for at least 4 days at 4°C.

Introduction

An important objective in cancer chemotherapy is the selective delivery of antineoplastic agents to a local tumour and more particularly to metastases in the body. If successful, this would lead to an improvement of the therapeutic index of highly active but toxic agents and a decrease in side-effects and adverse reactions.

Many approaches for the achievement of drug targeting have been reported, including the use of colloidal systems as drug carriers (Widder et al., 1982; Juliano, 1981; Illum and Davis, 1982). Normally, small colloidal particles are taken up by the reticuloendothelial system and subsequently deposited primarily in the liver and spleen. However, this has to be avoided if other organ sites are the intended targets. In general, the uptake of particles by the liver can be reduced by using small particles coated with non-ionic surface-active agents (Illum and Davis, 1983) or by

blocking the reticuloendothelial system by prior administration of large doses of placebo colloid. Particles less than 100 nm are believed to be able to leave the systemic circulation through fenestrations in the endothelial cells of the blood vessels in the liver, spleen and bone marrow, thereby reaching the extravascular sites. There is a suggestion that capillaries in tumour regions may also have greater permeability than normal because of tissue inflammation (Grislain et al., 1983).

In the present study we have brought together two potential approaches for drug targeting; nanoparticles and monoclonal antibodies. Polyalkylcyanoacrylate nanoparticles are able to sorb efficiently a large variety of drugs (Kreuter et al., 1983). They are non-toxic and biodegradable, the degradation being dependent upon the alkyl chain length (Couvreur et al., 1980; Kante et al., 1982). Recently Grislain et al. (1983) has shown that polybutylcyanoacrylate nanoparticles with a size around 100 nm are able to pass through the vascular endothelial cells and reach and accumulate in tumour tissue (i.e. subcutaneously grafted Lewis Lung carcinoma). In addition, the nanoparticles apparently also reached metastases in the lungs. However, the nanoparticles were not totally selective and significant quantities were found in other tissues including the liver.

The potential of monoclonal antibodies for radioimmunodetection of tumours and metastases and as possible carriers for antitumour agents has been considered. Monoclonal antibodies are produced which recognize antigens associated with human tumours including osteogenic sarcoma (Embleton et al., 1981). Pimm et al. (1982a) have shown that monoclonal antibodies to osteogenic sarcoma are preferentially taken up into tumour tissue compared with muscle, bone or visceral organs; the tumour uptake being a high as 20% of the total body count of radioactivity.

Although drug–antibody conjugates have the ability to target to tumour sites, the linking of the drug can be a difficult procedure that may result in inactivation of the drug and/or the antibody (Pimm et al., 1982b). Also the amount of antibody deposited must be sufficient to provide an adequate concentration of the attached therapeutic agent. A colloidal system, i.e. nanoparticles with a large carrier capacity coated with monoclonal antibodies, would be an interesting alternative. Thus in the present study we have investigated the adsorption of monoclonal antibodies to polyalkylcyanoacrylate nanoparticles and the immunospecific targeting to tumour cells in vitro. Polyhexylcyanoacrylate nanoparticles have been prepared using Dextran 70 as a stabilizer. The particles were coated with monoclonal antibodies and a fluorescent label and the binding of the conjugates to target cells in vitro was followed by means of flow cytofluorimetry.

Materials and Methods

Media

Phosphate-buffered saline (pH 7.4) (PBS), Hank's balanced salt solution (HBSS)and Eagle's minimum essential culture media (EMECM) were prepared using chemicals of reagent grade. In some experiments 5% calf serum (CS) was added to the culture medium.

Cells

The target cells used in the in vitro nanoparticle cell binding assay were the human osteogenic sarcoma cell lines 788T and T24. The cell lines were grown in vitro as monolayers in EMECM supplemented with 10% foetal calf serum. For the assay cells were harvested with trypsin 0.25% (w/v) in HBSS (Flow Laboratories, Victoria Park, Heatherhouse Road, Irvine, Ayrshire, U.K.), and washed 3 times in serum-free HBSS in which they were resuspended. Monodisperse cell suspensions were obtained following aspiration through a 21-gauge hypodermic needle to disperse cell clumps and filtration through a 300-gauge mesh (50 μm pore diameter).

Antibodies

The anti-osteogenic sarcoma monoclonal antibodies 791T/36 (Batch B3376 and B3493), 791T/48 (Batch B3448) that recognize different epitopes on 788T osteogenic sarcoma cells but do not bind to T24 cells, were prepared as supernatants from in vitro cultured cells as described by Pimm et al. (1982a).

Fluorescein isothiocyanate (FITC)-labelled 791T/36 antibody (Batch LR8) was prepared as described by Pimm et al. (1982a).

Normal mouse IgG was obtained commercially (Miles Laboratories, Research Products Division, Slough, U.K.; Mouse IgG Lyo).

Preparation of polyhexylcyanoacrylate nanoparticles (NP)

Related studies in the literature (Biagchi and Birnbaum, 1981) suggest that immunoglobulins will be well adsorbed to a hydrophobic surface. Therefore the normally employed non-ionic surfactant as stabilizer for nanoparticle preparation was replaced using the less hydrophilic material Dextran 70.

1 ml of hexyl-2-cyanoacrylate (Sichel Werke, Hannover, F.R.G.) was dispersed at 4°C in 100 ml of normal saline containing 0.01 N HCl and 1% (w/v) Dextran 70 under mechanical stirring. After polymerization was complete the resultant suspension was adjusted to pH 7.0 with 1 M NaOH and stored at 4°C. All solutions used, with the exception of the monomer, were sterile and aseptic techniques were used throughout the preparation.

The diameter of the nanoparticles, as measured by photon correlation spectroscopy (Malvern Instruments) (Berne and Pecora, 1977) was about 170 nm.

Fluorescein conjugation of bovine serum albumin (BSA-Fl)

In order to detect antibody–antigen binding the nanoparticles were labelled with fluorescent bovine serum albumin. The conjugation of fluorescein to bovine serum albumin was performed following a slightly modified method described by Johnson et al. (1978). 1 ml of BSA at 20 mg/ml in bicarbonate buffer pH 9.5 containing 0.9% (w/v) NaCl was added to a glass bijoux onto the walls of which had previously been dried 800 μg fluorescein isothiocyanate in 800 μl acetone. The mixture was gently rotated on a roller mixer for 1 h in the dark at room temperature. Free fluorescein was separated from the fluorescein-conjugated BSA in the mixture by gel filtration on a Sephadex PD10 (Pharmacia, Hounslow, U.K.) column equilibrated with PBS containing 0.02% (w/v) NaN_3. Following the void volume 2.75 ml of the eluate

containing the conjugate in PBS 0.02% (w/v) NaN_3 was collected, and assuming a 90% recovery of protein the concentration was estimated to be 6.5 mg/ml. The ratio of optical density (OD_{280} : OD_{495}) of the preparation was determined to be 1.7, using a Unicam UV spectrophotometer. The BSA-Fl preparation in PBS 0.02% (w/v) NaN_3 was stored at 4°C in the dark.

Radioiodination of 791T/36 monoclonal antibody

In order to measure the adsorption isotherm of monoclonal antibody nanoparticles, the antibody (791T/36) was labelled with ^{125}I using the iodogen method (Fraker and Speck, 1978). For labelling 100 μg of Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril, Pierce Chemicals, Chester, U.K.) in 0.3 ml methylene chloride, were evaporated to dryness, under nitrogen in conical polypropylene 'microfuge' tubes (Sarstedt, Leicester, U.K.). The 791T/36 antibody was diluted to 1 mg/ml in PBS and 0.5 ml introduced into the 'microfuge' tube followed by 3.3 μl $Na^{125}I$ (200 μCi) (for protein iodination, Radiochemical Centre, Amersham, U.K.). The contents of the tubes were mixed by repeated aspiration with a pipette every 2.5 min over a period of 15 min. The iodination reaction was stopped by rapid 'desalting' through a Sephadex PD10 column equilibrated with PBS 0.02% (w/v) NaN_3. Following the void volume 0.5 ml fractions were collected, a peak of radioactivity, corresponding to the conjugate, was found in fractions 2–5 which were pooled and stored at 4°C. The preparations had an activity of 1.6×10^8 cpm/ml. The stability of the labelling was investigated and it was found that 2 weeks after the preparation 97% of the radioactivity was precipitable with 5% (w/v) trichloroacetic acid (TCA).

Coating of nanoparticles and adsorption isotherms

Proteins and conjugates were adsorbed onto the nanoparticles by overnight incubation (equilibrium conditions) at 1/20 dilution of the nanoparticle preparation in 1 ml PBS 0.02% (w/v) NaN_3 containing 1 mg/ml of either 791T/36 antibody or mouse immunoglobulin and approximately 0.5 mg/ml of BSA-Fl.

In studies where it was desirable to monitor the association of the 791T/36 antibody with the nanoparticles (e.g. adsorption isotherm), the adsorption mixture additionally contained 20 μl of [^{125}I]791T/36 antibody (i.e. 0.02 mg/ml final concentration). The mixture was centrifuged for 2 h at 180,000 *g* (MSE Superspeed 65, 10 × 10 ml angle rotor) and the activity in the supernatant measured using a gamma counter (WILJ Gamma Counter 2001) with appropriate controls to take account of the adsorption of antibody to the surfaces of the centrifuge tubes.

Gel filtration of coated nanoparticles

The coated nanoparticles were separated from the adsorption mixture by gel filtration on Sepharose CL-4B columns (Pharmacia, Hounslow, U.K.). A 17 cm long by 1.5 cm diameter volume of the gel equilibrated with PBS 0.02% (w/v) NaN_3 was loaded with 0.8 ml of the nanoparticle adsorption mixture. The nanoparticles were eluted with PBS 0.02% (w/v) NaN_3 at a flow rate of 25 ml/cm^2/h and 1 ml fractions were collected. The elution of nanoparticles and protein from the column was monitored continuously by UV absorption (LKB Uvicord II).

A peak containing nanoparticles was routinely obtained in fractions 16–19 inclusive, these were pooled and normally used in an assay on the same day unless otherwise stated.

Assay for specific binding of coated nanoparticles to tumour cells

The binding of coated nanoparticles to tumour cells was measured by flow cytofluorimetry using a Fluorescence Activated Cell Sorter, FACS IV (Becton Dickinson, Sunnyvale, CA, U.S.A.). The specificity of the binding was assessed by inclusion in the assay of antigenically inappropriate target cells and the capacity of pretreatment of the target cells with antibody to block subsequent binding of the nanoparticles. In the assay 10^5 tumour cells at 10^6/ml HBSS were incubated with 100 μl of antibody (normally 791T/36 at 100 μg/ml PBS) in a 'microfuge' tube for 0.5 h at 4°C. The cells were then washed 3 times, each with 1 ml of ice-cold HBSS, with sedimentation by 5 s centrifugation in a 'microfuge' (Beckman Microfuge B). The supernatant was removed by aspiration and the cells resuspended in the remaining droplet using a Whirlimixer (Fisons). 100 μl of the coated nanoparticle preparation was added to the cells and after 0.5 h at 4°C in the dark the cells were washed once and resuspended in 0.5 ml of ice-cold HBSS. The mean fluorescence intensity of the cells was determined by flow cytofluorimetry, using a FACS IV. Excitation was at 120 mW at 488 nm from an argon ion laser and fluorescence collected via a 10 nm band-pass filter centred at 515 nm. Linear amplification was used to quantitate mean fluorescence intensity and the fluorescence signals were also amplified logarithmically for direct comparison of fluorescence distribution over a wide range of fluorescence intensity. Included in the assay were controls for the specificity of the antibody for the cells. Following pretreatment cells were treated with fluorescein-labelled 791T/36 at 4.0 μg/ml in PBS or PBS as control, instead of coated nanoparticles.

Results and Discussion

Gel filtration of the nanoparticle adsorption mixture gave elution profiles with two distinct peaks (Fig. 1). The first of these corresponded to the nanoparticles and the second to the adsorption mixture when each was run separately under identical conditions. Fig. 1 shows the elution profile and the radioactivity associated with the eluted fractions of a nanoparticle adsorption mixture containing 791T/36 antibody, [^{125}I]791T/36 antibody and BSA-Fl. The inclusion of the radiolabelled antibody permitted estimation of the percentage of antibody in the mixture eluted with the nanoparticles. 81% of the radioactivity of the nanoparticle adsorption mixture loaded onto the column was recovered in the eluted fractions, 36% of which was considered to be eluted with the nanoparticles in fractions 15–24 and the remaining 64% in the second peak in fractions 25–52 inclusive. Fractions 16–19 inclusive were routinely pooled to be the coated nanoparticle preparation, and 73% of the radioactivity associated with the nanoparticle peak was present in these pooled fractions. Thus about 20% of the original antibody in the adsorption mixture was present in

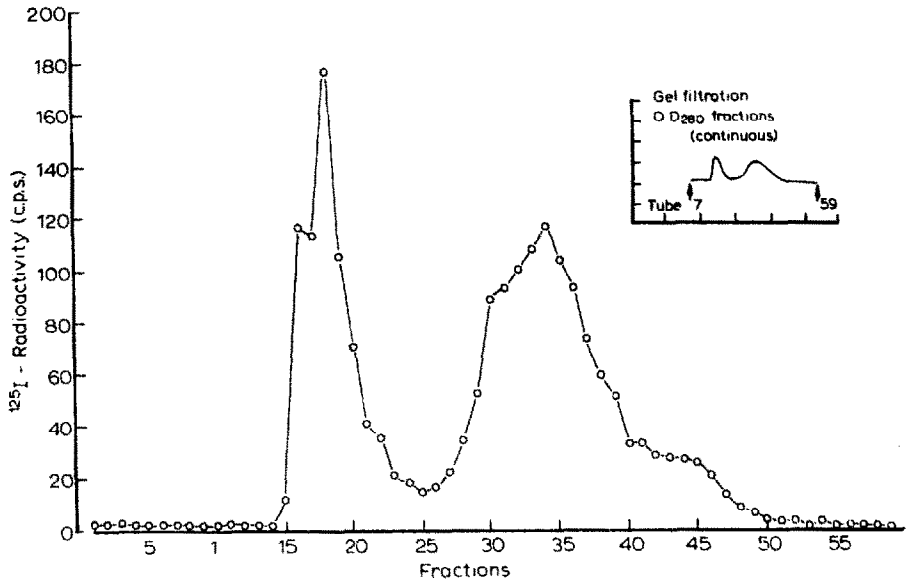

Fig. 1. Gel filtration elution profile of antibody-coated polyhexylcyanoacrylate nanoparticles and the adsorption mixture containing 791T/36 antibody [^{125}I]791T/36 antibody and BSA-Fl.

these pooled fractions. The stability of the binding of the antibody to the nanoparticles was inferred from a separate study in which 12% of the antibody in the adsorption mixture was present in the pooled eluted fractions 15–19. In this study the nanoparticles in the preparation were sedimented by centrifugation at 180,000 *g* for 2 h at 4°C on various days after gel filtration. The fraction of the radioactivity of the preparation (pooled eluted fraction) found in the supernatant was considered to be a measure of the unadsorbed antibody in the preparation. Initially, 0.85% of the antibody in the preparation was found in the supernatant, by days 4 and 7 this had increased to 2.0% and 4.5%, respectively. However, it should be emphasized that these values indicating good stability of binding may be underestimates since significant adsorption of free antibody to the walls of the centrifuge tubes has been shown to occur (in the order of 7% of initial concentrations).

The adsorption isotherm studies showed that as expected (Biagchi and Birnbaum, 1981) the isotherm was Langmuirian in nature and the total binding capacity was 1460 μg antibody/mg nanoparticles. Calculations made using surface area values based on a monodisperse distribution of nanoparticles of size 170 nm in diameter and the estimated area of 38 nm^2 per adsorbed immunoglobulin molecule (monoclonal antibody) at a pH value of 7 (Biagchi and Birnbaum, 1981) allowed an estimation of the theoretical maximum number of monoclonal antibodies on each individual nanoparticle to be 2000.

The results of the studies investigating the capacity of antibody coated nanoparticles to bind specifically to tumour cells in vitro are shown in Tables 1–3. In each study the specificity of the 791T/36 antibody for 788T cells but not T24 cells was confirmed by the findings that the mean fluorescence intensity of 788T cells but not

TABLE 1

EFFECT OF CALF SERUM (CS) ON SPECIFIC BINDING OF COATED NANOPARTICLES (NP) TO TARGET CELLS

Tube	Cells	Pretreatment	Treatment (±5% CS)	Mean fluorescence intensity (arbitrary units)
O-1	788T	Media	Media	6.1
O-2	788T	Media	Fl-Ab	288.4
O-3	788T	Ab	Fl-Ab	109.2
O-4	T24	Ab	Media	9.2
O-5	T24	Media	Fl-Ab	31.8
O-6	T24	Ab	Fl-Ab	23.3
NP-1	788T	Media	Ab-NP-BSA-Fl(+)	4.5
NP-2	788T	Media	Ab-NP-BSA-Fl(−)	32.3
NP-3	788T	Ab	Ab-NP-BSA-Fl(+)	4.8
NP-4	788T	Ab	Ab-NP-BSA-Fl(−)	11.4
NP-5	T24	Media	Ab-NP-BSA-Fl(+)	7.6
NP-6	T24	Media	Ab-NP-BSA-Fl(−)	14.7

Media: PBS with 0.02% (w/v) NaN_3, 5% CS. Ab: 100 μg/ml 791T/36 in PBS 0.02% NaN_3 5% CS (origin Batch B3376). Fl-Ab: 20 μg/ml fluorescein-labelled 791T/36 PBS in 0.02% NaN_3, 5% CS (origin Batch B3315).

TABLE 2

SPECIFIC BINDING OF ANTIBODY (Ab)-COATED NANOPARTICLES (NP) TO TARGET CELLS

Tube	Cells	Pretreatment	Treatment	Mean fluorescence intensity (arbitrary units)
O-1	788T	Media	PBS	7.6
O-2	788T	Media	Fl-Ab	199.5
O-3	788T	Ab	Fl-Ab	46.8
O-4	T24	Media	PBS	8.5
O-5	T24	Media	Fl-Ab	18.0
O-6	T24	Ab	Fl-Ab	16.6
NP-1	788T	Media	Ab-NP-BSA-Fl	43.9
NP-2	788T	Media	Ig-NP-BSA-Fl	12.9
NP-3	788T	Ab	Ab-NP-BSA-Fl	13.9
NP-4	788T	Ab	Ig-NP-BSA-Fl	13.9
NP-5	T24	Media	Ab-NP-BSA-Fl	17.9
NP-6	T24	Media	Ig-NP-BSA-Fl	17.7
NP-7	T24	Ab	Ab-NP-BSA-Fl	14.4
NP-8	T24	Ab	Ig-NP-BSA-Fl	16.3
NP-9	788T	PBS	Ab-NP-BSA-Fl	37.3
NP-10	788T	PBS	Ig-NP-BSA-Fl	12.3

Media: 100 μg mouse Ig/ml PBS (origin Miles). Ab: 100 μg 791T/36/ml PBS (origin B3376). Fl-Ab: 4.0 μg fluorescein-labelled 791T/36/ml PBS (origin LR8)

T24 cells was greatly increased following their treatment with fluorescein-conjugated antibody. Furthermore, the increase was diminished if the cells had first been treated with non-conjugated antibody.

The treatment of cells with antibody is normally done in the presence of 5% CS to reduce the non-specific adsorption of antibody by the cells. While the presence of CS during treatment of cells with antibody-coated nanoparticles might be similarly desirable to reduce the non-specific binding of antibody-coated nanoparticles to the cells, it could also have a deleterious effect by displacing the antibody bound to the nanoparticles. Thus in the first study the influence of the presence of CS on the capacity of nanoparticles coated with antibody and fluorescein-conjugated BSA to bind specifically to cells was investigated. It was found that in the presence of CS the fluorescence intensity of both 788T cells and T24 cells incubated with the coated nanoparticles was similar to those incubated in media alone (Table 1). However, in the absence of CS, although the fluorescence intensity of T24 cells incubated with

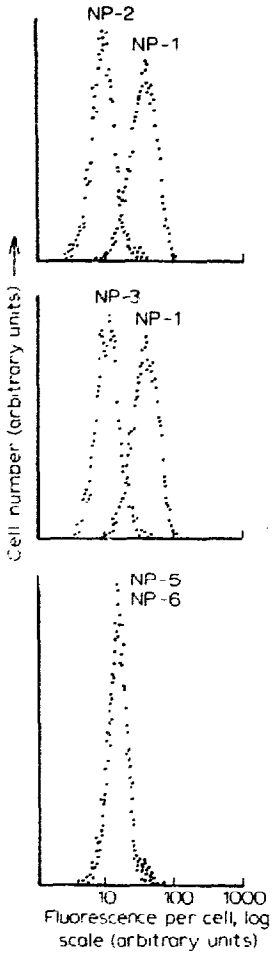

Fig. 2. Graphs of flow cytofluorimetric analysis results from Table 2 showing the log normal fluorescence distribution of 788T and T24 tumour cells derived from cell cultures pretreated with media or antibody and incubated with either Ab-NP-BSA-Fl or Ig-NP-BSA-Fl nanoparticle conjugates.

coated nanoparticles was increased, indicating some non-specific binding of nanoparticles and/or free fluorescein-labelled BSA to the cells the fluorescence intensity of the 788T cells which had not been pretreated with antibody was increased to a higher level. The results suggest that in the absence of CS, specific binding of antibody-coated nanoparticles to cells is demonstrated.

After sedimentation by ultracentrifugation of nanoparticles coated with radiolabelled antibody, only 3% of the radioactivity was found in the supernatant but if the sedimentation was done in the presence of 10% CS then the amount of radioactivity in the supernatant was increased to 12%. While this could imply that the CS displaces antibody from the nanoparticles it should be remembered that since the centrifuge tubes were shown to adsorb significant amounts of free antibody, the greater radioactivity in the supernatant containing 10% CS could also be due to reduced adsorption of free antibody by the centrifuge tube due to the presence of CS.

The capacity of nanoparticles freshly coated with antibody and fluorescein-conjugated BSA to bind specifically to target cells in vitro, in the absence of CS, was confirmed in further studies. Antibody-coated nanoparticles bound specifically to 788T target cells but not T24 cells and normal immunoglobulin-coated nanoparticles failed to bind to either cell type. Furthermore the binding of antibody-coated nanoparticles to 788T cells could be inhibited by pretreatment of the cells with antibody but not with media containing a similar concentration of normal im-

TABLE 3

RETENTION OF CAPACITY OF ANTIBODY (Ab)-COATED NANOPARTICLES (NP) TO BIND SPECIFICALLY TO TARGET CELLS

Tube	Cells	Pretreatment	Treatment	Mean fluorescence intensity (arbitrary units)		
				Day 0	Day 4	Day 7
O-1	788T	Media	PBS	3.7	7.4	5.1
O-2	788T	Media	Fl-Ab	187.6	283.7	314.3
O-3	788T	Ab	Fl-Ab	98.5	361.0	50.6
O-4	T24	Media	PBS	4.6	6.1	7.5
O-5	T24	Media	Fl-Ab	13.1	11.3	15.4
O-6	T24	Ab	Fl-Ab	11.0	10.5	8.3
NP-1	788T	Media	Ab-NP-BSA-Fl	22.0	15.3	12.4
NP-2	788T	Media	Ig-NP-BSA-Fl	6.8	9.0	7.1
NP-3	788T	Ab	Ab-NP-BSA-Fl	9.1	16.5	11.4
NP-4	788T	Ab	Ig-NP-BSA-Fl	6.1	9.1	12.0
NP-5	T24	Media	Ab-NP-BSA-Fl	8.7	8.7	9.5
NP-6	T24	Media	Ig-NP-BSA-Fl	7.7	9.1	9.6
NP-7	T24	Ab	Ab-NP-BSA-Fl	8.0	9.4	8.8
NP-8	T24	Ab	Ig-NP-BSA-Fl	7.2	8.1	9.5

Media: 100 μg mouse Ig/ml PBS (origin Miles). Ab: 100 μg 791T/36 or 791T/48/ml PBS. Day 0 and Day 7: 791T/36 (origin batch B3376 and Batch B3493) Day 4: 791T/48 (Batch B3448). Fl-Ab: 4.0 μg fluorescein-labelled 791T/36/ml PBS (Origin LR8).

munoglobulin (Table 2 and Fig. 2). The capacity of the coated nanoparticles to bind specifically to the target 788T cells was retained for up to 4 days after coating of the nanoparticles but by 7 days specific binding of the nanoparticles to the cells was no longer demonstrable. It was also shown that pretreatment of the cells with antibody recognizing a different epitope on the 788T cells to that recognized by the antibody coating the nanoparticles did not affect the specific binding of the coated nanoparticles to the cells (Table 3).

Conclusions

The results show that monoclonal antibody is well adsorbed onto the surface of the polyhexylcyanoacrylate nanoparticles and that nanoparticles thus coated interact in a specific way with antigenic tumour cells in vitro. The adsorbed monoclonal antibody retains its immunospecificity for at least 4 days at 4°C.

The adsorption of an antibody molecule onto a nanoparticle may occur via either its antigen binding sites (Fab) or the Fc portion of the molecule. If the surface of the particle is hydrophilic the specific antibodies tend to bind to the surface through the two Fab groups (antigen binding sites) with the Fc portion protruding outwards thus forming a more hydrophobic outer surface (van Oss et al., 1975). In contrast if the surface of a particle is hydrophobic the Fc portions can bind to the surface thereby leaving the Fab binding sites free to interact with antigenic cells (Fig. 3).

In the present study the specific interaction between antibody-coated nanoparticles and antigenic cells demonstrates conclusively that the 791T/36 monoclonal antibody can be adsorbed via the Fc fraction. It is estimated that each nanoparticle can carry an average of 2000 monoclonal antibodies.

The specific interaction between nanoparticles coated with monoclonal antibody and antigenic tumour cells in vitro indicates that this system might be useful for drug targeting in vivo. Studies using nanoparticles carrying an anticancer drug and coated with an appropriate monoclonal antibody for targeting to tumours implanted in mice are in progress. However, the results that have been obtained using calf serum as part of the medium for antigen–antibody interactions in vitro suggest that there is the possibility of competitive displacement of the adsorbed antibody. Serum

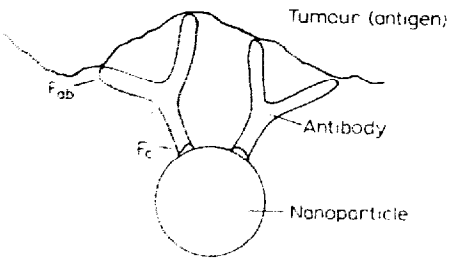

Fig. 3. Schematic diagram of the attachment of monoclonal antibody to a nanoparticle.

contains a large number of components that could cause such displacement. Clearly if this is significant during the period of targeting, alternative methods will need to be investigated, i.e. covalent binding for the linking of the antibody to the particle surface.

The loss of immunospecificity with time may be caused by surface denaturation (unfolding) of the antibody. The use of deep freeze and freeze-drying techniques to extend the viability of the nanoparticle–antibody system will be explored.

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