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Monoclonal antibodies covalently coupled to polymethacrylic nanoparticles: in vitro specific targeting to human T lymphocytes

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Summary

Previously, methacrylic copolymer nanoparticles have been used as drug-carriers for passive targeting. With the aim of a modification in the natural distribution pattern of the nanospheres to the reticulo-endothelial system, we present the covalent linking of monoclonal antibodies to the nanoparticles and the active targeting of these immunonanospheres in vitro. To perform this technique, a model was chosen: a CD3 monoclonal antibody (an anti-peripheral human T lymphocyte antibody) and peripheral blood mononuclear cells as targets. Labeling of cells was observed by fluorescence microscopy and by scanning electron microscopy.

Introduction

Passive targeting, using small colloidal particles as drug-carriers generally leads, after intravenous administration, to their uptake by the mononuclear phagocytes of the reticuloendothelial system predominantly in the liver and spleen and by circulating blood monocytes (Kreuter et al., 1979; Arturson et al., 1983; Grislain et al., 1983; Sjoholm and Edman, 1979; Illum et al., 1982; Poste, 1983). It is now well known that passive targeting mainly depends on particle size and shape (Illum et al., 1982; Illum and Davis, 1982), surface charge and surface characteristics (hydrophilicity/hydrophobicity) (Illum et al., 1982; Illum and Davis, 1982; Kreuter, 1983; Leu et al., 1984).

Active targeting refers to a modification in the natural distribution pattern of the drug-carrier, using for example monoclonal antibody-particle systems. Of course it is possible to use antibodies themselves as drug-carriers, i.e. to bind drugs directly to antibodies without using polymers as intermediates (Rowland, 1983; Arnon and Hurwitz, 1983; Ghose et al., 1983); nevertheless there are some problems: the linking of the drug to immunoglobulins may result in a loss of antibody activity and/or drug (Hurwitz et al., 1975; Davies and O'Neill, 1974; Pimm et al., 1982), and the binding of anticancer drug molecules to monoclonal antibodies is often limited. For these reasons, the use of methacrylic copolymer nanoparticles as an intermediate carrier has been studied. These nanospheres have been used in vivo as

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drug-carrier for passive targeting (Rolland et al., 1986a; Rolland, 1987) and this article describes the covalent linking of monoclonal antibodies to the nanoparticles, then in vitro, the active targeting of drug-carrier-antibody. To perform this technique a model was chosen: a CD3 monoclonal antibody (an anti-human T mature lymphocyte antibody) and peripheral blood mononuclear cells (PBMCs) as targets.

Materials and Methods

Preparation of the nanoparticles

Referring to a method described by Molday et al. (1975), Rembaum et al. (1979) and Rembaum and Dreyer (1980), the nanoparticles were prepared by emulsion copolymerization of methacrylic monomers, purified before use by distillation as indicated elsewhere (Rolland et al., 1986b). The following monomers, methylmethacrylate (1.4 ml), 2-hydroxypropylmethacrylate (0.75 ml), methacrylic acid (0.25 ml) and ethylene glycol dimethacrylate (0.10 ml) (Merck) were introduced, with stirring, into 95 ml distilled water; the reaction was then chemically started by adding 10 mg of a free radical initiator (potassium persulfate, Merck) and the copolymerization was maintained at 90 °C for 1 h. After filtration (glass filter 10–20 μm), the suspension was dialysed to remove the remaining monomers and other ionic impurities. The quality of this purification was controlled by dosage of the residual monomers by high-performance liquid chromatography (Rolland et al., 1986c). The size of the nanoparticles was assessed using a Coulter Nano-Sizer (Coultronics) and their morphology and internal structure were observed by scanning and transmission electron microscopy. The concentration of the particles in suspension was based on dry weight analysis, and the particles were numbered theoretically. The presence of hydroxyl and carboxyl groups in the nanospheres was detected by nuclear magnetic resonance and infrared spectrometry (Rolland et al., 1986d) and hydrogen ion titration measurements indicated the number of carboxyl groups on the surface of each particle. The molecular weight of the methacrylic copolymers was estimated by liquid exclusion chromatography. The surface charge of the nanospheres was assessed in distilled water at 25 °C.

Production of monoclonal antibodies (M.Abs.)

A Balb/c mouse was immunized twice intraperitoneally with PBMCs from a normal donor; 4 days after the boost, the spleen was harvested under sterile conditions. The fusion was performed in accordance with the technique of Galfre et al. (1977) and described by Bourel et al. (1984). Briefly, 100×10^6 splenocytes and 10×10^6 SP_2O myeloma cells were fused with PEG (Serva 4000). After the fusion, the cells were transferred into 96 well microculture plates in a selective Hypoxanthin Azaserin medium; 10 days later at 37 °C, the supernatants were collected and tested for the detection of M.Abs. Lymphocytotoxicity and indirect immunofluorescence tests were performed with immunizing cells and panels of miscellaneous human cell suspensions: T and B lymphocytes, EBV cell lines, and malignant cell proliferations. The selected hybridomas were cloned by limiting dilution and cryopreserved in liquid nitrogen. The antibodies were produced either by hybrid cultures or by mouse tumour induction. Isotypes of the secreted antibodies were determined by Ouchterlony technique. X.35, a CD3 monoclonal antibody, was selected for this study.

Preparation of the immunonanospheres

Fluorescent nanoparticles. The suspension of nanoparticles was adjusted to pH 8.0 (0.01 M CH_3COONa; 1 N NaOH) and an aqueous solution of ethidium bromide (Merck) was added, with stirring at 20 °C, to give a final concentration of 2.5 mM with a 100% adsorption yield. The fluorescent nanoparticles were then dialysed against distilled water for 24 h.

Modified nanoparticles. 1,7-Diaminoheptane (Merck) was bound to the particles using the carbodiimide reaction, to make a spacer (Hoare and Koshland, 1967; Rembaum et al., 1976; Yen et al., 1979). 4 ml of 0.1 M diaminoheptane were mixed with 36 ml fluorescent nanoparticles and the suspension was adjusted to pH 7.0 with 1N HCl. Then, 133 mg of 1-ethyl-3(3-dimethyl

aminopropyl)carbodiimide (Merck) were added, with stirring, to the cold colloidal suspension. After 4 h at 4°C, the suspension was purified by dialysis against 1 liter of 0.1 M NaCl for 2 h and 4 liters of PBS (phosphate-buffered saline) for 10 h. Derivatized nanospheres were stored at 4°C in the dark until monoclonal antibody coupling.

Monoclonal antibody–nanoparticle linkage. X.35 M.Ab. was coupled to the derivatized nanoparticles by covalent linkage (Molday et al., 1975; Rembaum et al., 1976; Rembaum and Dreyer, 1980) using the glutaraldehyde method in two steps (Bowes and Cater, 1966; Otto et al., 1973). 0.3 ml of an aqueous glutaraldehyde solution (25% glutaraldehyde, Sigma) was dropped, with stirring, to 5.7 ml derivatized particles. After stirring for 1 h at 20°C, the suspension was dialysed against PBS in the cold for 24 h. 600 μl of purified M.Abs. (25 μg/ml) were added with stirring to 6 ml glutaraldehyde-activated nanospheres, and the linkage was made by incubation for 19 h at 4°C. Finally, the reaction was stopped by adding 1.2 ml of 0.1 M glycine aqueous solution (Fluka). After 5 h at 4°C, the final suspension was dialysed against 4 changes of 1 liter each of PBS at 4°C over a 48 h period.

Purification of the immunonanospheres. M.Abs. bound to fluorescent nanoparticles were separated from unbound antibodies by gel filtration on Sepharose 4B (Pharmacia). 500 μl of immunonanospheres were loaded onto a column and eluted with PBS. The adsorption was detected in continuous flow using a UV photometer (240 nm). The first fluorescent fractions, corresponding to the immunonanoparticles, were collected and stored at 4°C in the dark until use.

Labeling of cells

PBMCs were isolated from whole blood by Ficoll gradient centrifugation. The cells were labeled, in one step, by adding the fluorescent immunonanospheres. 0.5×10^6 PBMCs were incubated, at 20°C, in microtubes with 100 μl of various dilutions of the immunonanoparticles. After 1 h, the cells were washed 3 times by centrifugation with PBS to eliminate the unbound nanoparticles. The labeled cells were observed by fluorescence microscopy and by scanning electron microscopy, as described previously (Rolland et al., 1987). Controls were performed with fluorescent nanoparticles, unbound, modified, or activated nanoparticles.

Results

Physicochemical properties of the nanoparticles

Using the Nano-Sizer and scanning electron microscopy, the size of the nanoparticles was assessed at 0.3 μm; they were spherical and their size distribution was homogeneous, without agglomerates (Fig. 1a). Their internal structure, observed using transmission electron microscopy after freeze-fracturing, appeared to be porous (Fig. 1b). The concentration of the particles in the suspension was valued to 18 mg/ml and calculated to be 2×10^{12} particles/ml suspension. Approximately 1.1×10^5 carboxyl groups were found on the surface of each particle. Their molecular weight was estimated to be 270,000. The surface charge was negative, with a low value of about −20 mV.

The size of the immunonanospheres was unchanged (0.3 μm) and the absence of aggregates was seen.

Selection of the X.35 monoclonal antibody

During a fusion, a CD3 M.Ab., X.35, was selected for its strong labeling to T lymphocytes. The X.35 hybrid secreted IgG2a molecules at high concentrations: 10 μg/ml in culture supernatant and 8–10 mg/ml in ascites. The X.35 M.Ab. reacted with all the human peripheral T lymphocytes, with mature thymocytes, some Acute-T Lymphoblastic Leukemias and the majority of Chronic-T Lymphoid Leukemias. No reactivity was observed with B lymphocytes and monocytes. The human peripheral mononuclear cells, isolated by Ficoll centrifugation and used for the tests, were composed on average of 70% T lymphocytes, 15% B lymphocytes, 5% monocytes and 10% null cells. With X.35 M.Ab., 70% of PBMCs were labeled (indirect immunofluorescence test using goat anti-mouse Ig).

Purification of the immunonanospheres

As seen in Fig. 2, showing the elution profile of

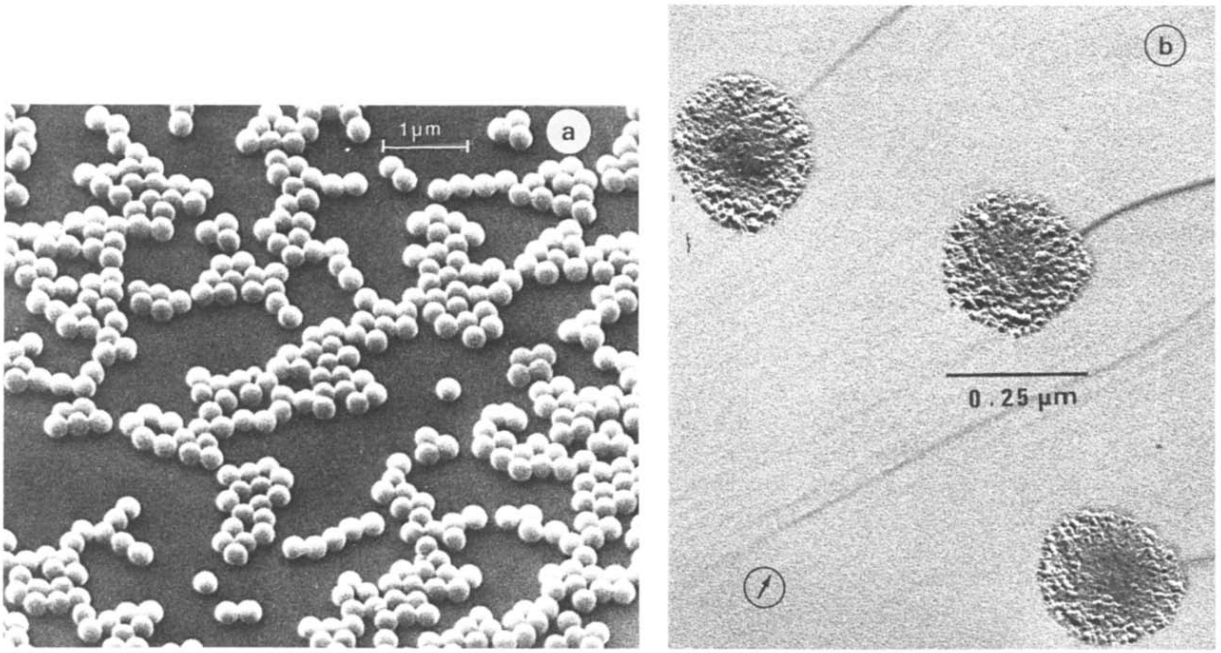

Fig. 1. Methacrylic copolymer nanoparticles synthesized by aqueous emulsion copolymerization. a: scanning electron micrograph. b: transmission electron micrograph after freeze-fracturing.

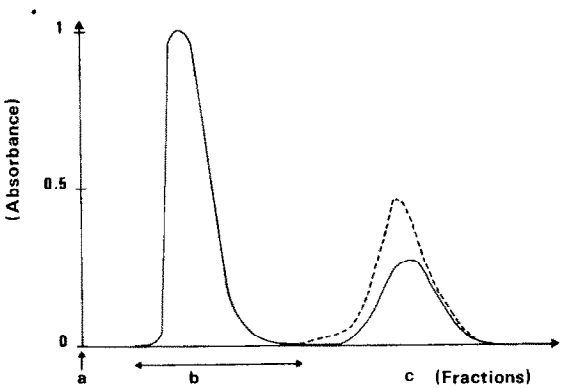

Fig. 2. Purification of the immunonanospheres by gel filtration on a Sepharose 4B column.

: (a) 500 µl of M.Abs-nanoparticles were loaded onto the column.
(b) collected fractions of purified immunonanospheres
(c) unbound X.35 monoclonal antibody.

------ pure X.35 M.Ab.

the immunonanospheres on Sepharose 4B, two distinct peaks were observed. The first peak corresponded to the fluorescent particles and the second to the unbound antibodies. The gel filtration allowed the purification of the immunonanospheres from free M.Abs. This purification was confirmed by an indirect immunofluorescence test using fluoresceinated goat anti-mouse Ig (data not shown).

Labeling of cells

Using purified immunonanoparticles, 70% PBMCs, corresponding to the T lymphocytes, were labeled while the presumed B-lymphocytes remained unlabeled (see Fig. 3). The monocytes were also fluorescent, because particles were phagocytosed as described previously (Rolland et al., 1987) (Fig. 3a and b). The distribution of the immunonanospheres was heterogeneous from one positive cell to another (Fig. 3b and d): some cells were weakly labeled while the majority was densely labeled. Besides, the repartition of the nanopar-

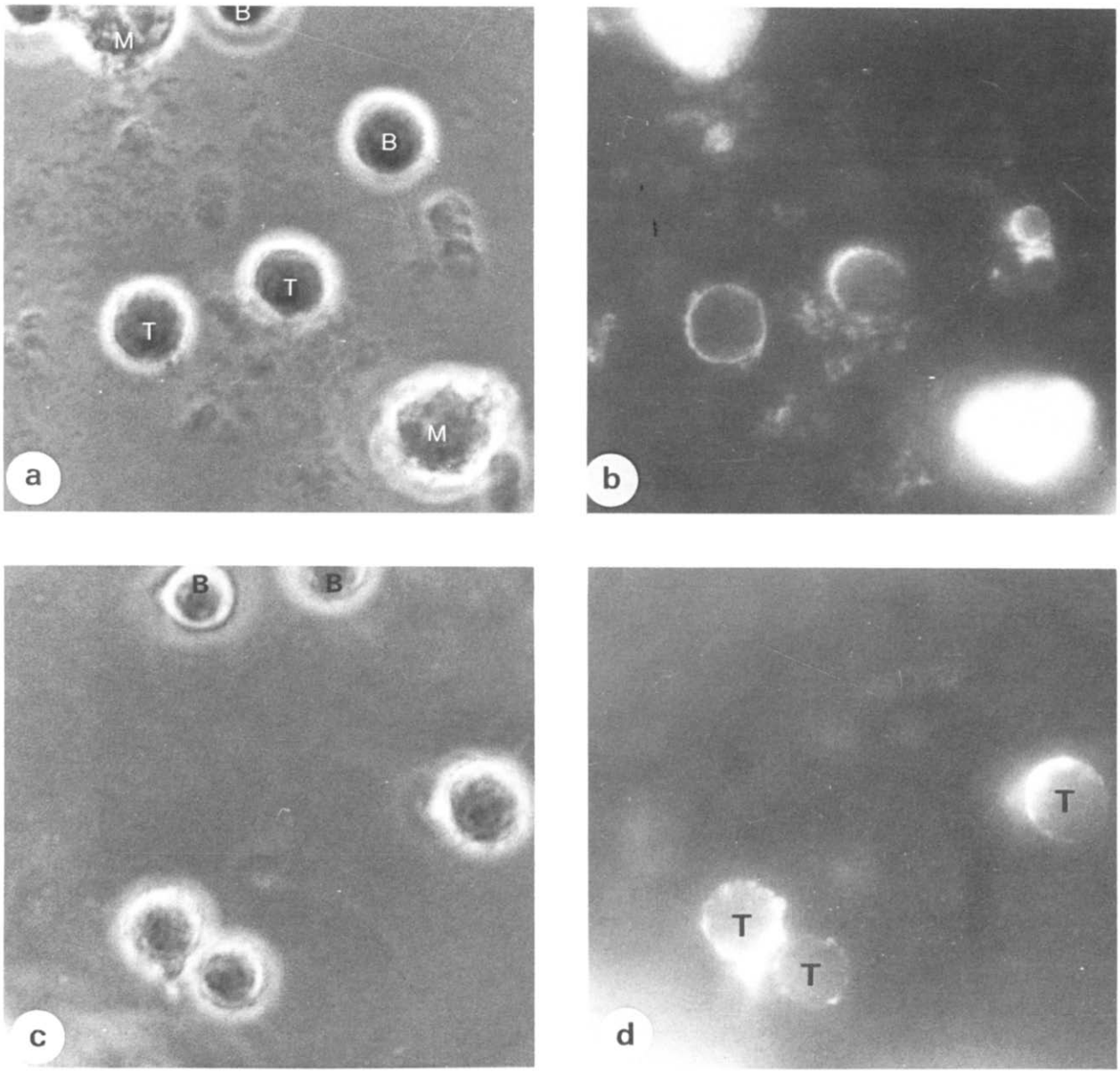

Fig. 3. Human T lymphocytes labeled with CD3 monoclonal antibodies coupled to fluorescent polymethacrylic nanospheres. M = monocytes; T = T lymphocytes; B = B lymphocytes. a and c: microscope photo of peripheral blood mononuclear cells (controls). b and d: fluorescent micrograph.

ticles on the surface of the cells was irregular: some cells were homogeneously covered with particles and other presented unlabeled areas. All the control tests were negative.

The results obtained by scanning electron microscopy were in accordance with the fluorescence data. Fig. 4 showed a labeled T lymphocyte and two negative cells identified as B lymphocytes.

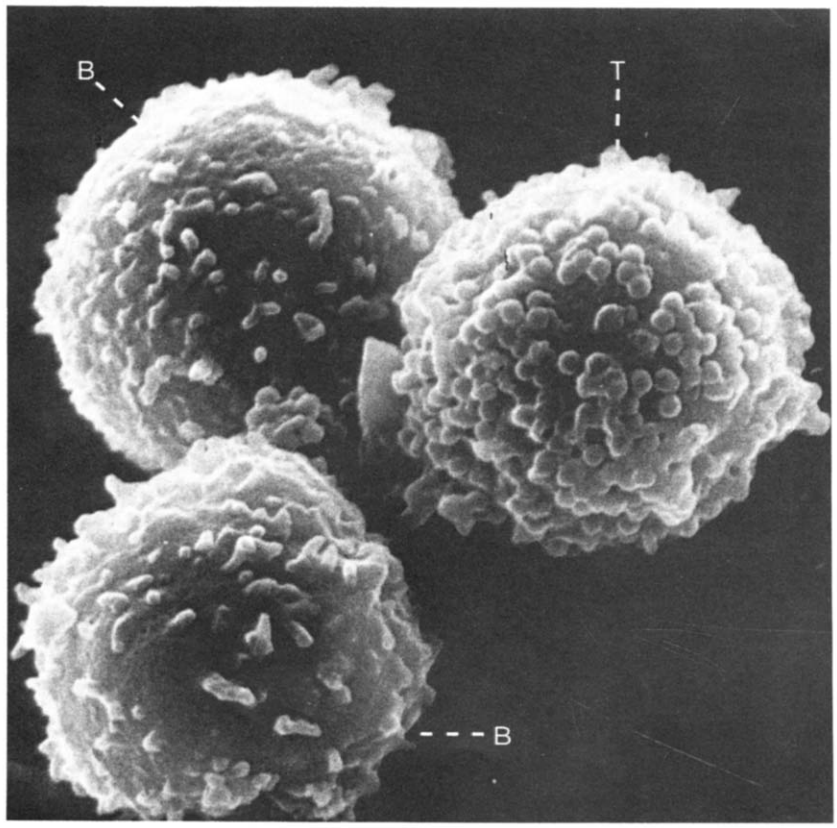

Fig. 4. Scanning electron micrograph of human T lymphocyte labeled with the immunonanospheres (unlabeled cells are believed to be B lymphocytes).

Discussion

To prove the possible active targeting of the nanospheres, using covalently bound monoclonal antibodies, a model was chosen: a CD3 monoclonal antibody (X.35), fluorescent methacrylic copolymer nanoparticles and PBMCs as targets.

Two kinds of assays were previously performed: (i) with passive adsorption of M.Abs. onto nanoparticles, no labeled cells were observed; and (ii) using the carbodiimide reaction (Yen et al., 1979) to bind directly M.Abs. to the nanospheres, without using a spacer, agglomerates of particles were always obtained, resulting in false positive reactions.

Covalent linking of the M.Abs. to the nanoparticles seemed to be necessary, because some authors showed that competitive displacement of M.Abs. by blood components could occur after intravenous injection of monoclonal antibodies only adsorbed onto nanoparticles (Barbet et al., 1981; Illum et al., 1983; Leu et al., 1984). So, immunonanospheres were prepared according to the technique described above (see Materials and Methods section) and purified before use by gel filtration on Sepharose 4B to avoid a competitive fixation between immunonanoparticles and free M.Abs. All the tests performed with the covalently bound CD3 M.Abs./nanospheres showed a specific fixation of the fluorescent particles on the T lymphocytes via the M.Abs. (fluorescence and scanning electron microscopy). Active targeting of the nanospheres proved in vitro, may be of interest because it shows that the covalent linkage of M.Abs. to particles is stable and that the specificity of the monoclonal antibody is maintained. So,

the natural distribution pattern of the nanoparticles, after intravenous administration, would perhaps be modified in vivo, by coupling monoclonal antibodies to the drug-carrier; for successful results (Bradfield, 1984), attempts would probably be necessary before to suppress the reticulo-endothelial clearance using pre-blocking agents, such as biodegradable colloids.

These immunonanospheres, consisting of monoclonal antibodies covalently bound to polymethacrylic nanoparticles with adsorbed drugs, would represent a second generation of drug-carriers, characterized by a specific cell targeting, corresponding to the specificity of the chosen monoclonal antibody.

We are initiating experiments to demonstrate the possibility of active targeting in vivo using monoclonal antibodies of different specificities. These immunonanospheres would find perhaps applications in cancer diagnosis and therapy.

In addition, the fluorescent immunonanoparticles represent a new biological reagent, allowing a direct labeling of cells that is not tedious and time-consuming like classical indirect immunofluorescence tests. This technique also allows a simultaneous double-labeling of cells, using two different immunoreagents, obtained by coupling two M.Abs. of different specificities to nanoparticles with two different fluorescent agents (ethidium bromide and fluorescein, for example). The visualization of the nanoparticles on the cell surface, by scanning electron microscopy, offers a new method for the study of the repartition of cell molecules and of phenomena such as capping.

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