The EMBO Journal Vol.2 No.10 pp.1655 – 1664, 1983

A monoclonal antibody to the heavy chain of clathrin

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Communicated by D.Louvard
Received on 27 May 1983; revised on 8 July 1983

Monoclonal antibodies have been raised to pig brain triskelions and one clone, DC41, was found to recognize the clathrin heavy chain by immunoblotting. However, both by immunofluorescence and immunoelectron microscopy, and in complete contrast to polyclonal anti-clathrin antibodies, monoclonal DC41 did not label either coated pits or coated vesicles anywhere in the cell. Instead it appeared to label the cell cytoplasm. These data suggest that DC41 recognizes a cytoplasmic form of clathrin, perhaps that form produced by uncoating of coated vesicles which is then ready to re-build another coated pit.

Key words: monoclonal antibodies/clathrin/heavy chain

Introduction

Coated vesicles mediate the transport of selected membrane and soluble components between different cellular membranes (for a review, see Pearse and Bretscher, 1981). The polyhedral coat is built up stepwise on the cytoplasmic side of the membrane giving a coated pit of gradually increasing curvature (Heuser, 1980). Eventually the coat forms a complete cage pinching off the trapped membrane to give a coated vesicle. Within minutes (Helenius *et al.*, 1980) the coat is removed enzymatically (Patzer *et al.*, 1982) leaving the vesicle to fuse specifically with another cellular membrane and the disassembled coat to diffuse back to a membrane to begin another round of coated pit formation.

The major protein of the coat is clathrin (Pearse, 1975) which comprises both heavy (mol. wt. 180 000) and light chains (mol. wt. 30 000 – 40 000 depending on the tissue and species) and the basic unit of assembly is the triskelion (Ungewickell and Branton, 1981), a flexible, three-armed structure comprising three heavy and three light chains. It is probable that the triskelion is the cytoplasmic intermediate liberated by vesicle uncoating and used to assemble fresh coated pits.

This clathrin cycle is of considerable structural and biochemical interest, yet few of the steps have been elucidated in any detail. There seems little doubt that the cycle involves considerable structural rearrangements of the clathrin molecule and parts of the molecule might only be exposed at certain stages. Antibodies to these parts might 'freeze' the cycle at one step permitting a more detailed study. With this in mind, we set out to raise monoclonal antibodies to pig brain triskelions. We report here studies on one clone which appears to discriminate between clathrin at different stages of its cycle.

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Results

Monoclonal antibody preparation

Hybridomas were screened by an immunoradiometric assay and out of 120 culture supernatants, 24 were positive for clathrin binding. These positive supernatants were then screened by indirect immunofluorescence and 11 gave punctate labelling in normal rat kidney (NRK) cells, whereas the rest gave no signal at all or labelling of cellular filaments. Three of the 11 supernatants gave a punctate pattern similar to that obtained using polyclonal anti-clathrin (see below) and the characterization of these hybridomas will be reported elsewhere; the rest gave punctate labelling throughout the cell cytoplasm. One of these, designated DC41, was cloned three times and the secretory product was characterized by metabolic labelling using [^{14}C]leucine. After analysis of the culture supernatants by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) the antibody appeared to be an IgM, an observation confirmed by immunoradiometric studies using antibodies specific for the IgM μ chain (data not shown).

Immunoblotting

Coated vesicles and triskelions from two different sources were fractionated by SDS-PAGE, transferred to nitrocellulose filters, and incubated with monoclonal DC41. The bound IgM was visualized using a second antibody coupled to horse radish peroxidase (HRP) and diaminobenzidine. As shown in Figure 1 (lanes 2 – 6), the antibody specifically recognized the heavy chain of clathrin but not the two light chains and none of the other proteins in the crude coated vesicle fraction. Apart from pig brain and rat liver clathrin we have also used DC41 to detect clathrin in cells derived from humans (WI38), dogs (MDCK), rats (NRK), rat kangaroo (PtK) and chickens (CEF) (not shown) showing that the epitope recognized is highly conserved. Although we have not as yet been able to use this antibody to detect clathrin in whole cell extracts, it does detect it in very crude preparations of coated vesicles (Figure 1, lanes 1,2).

The clathrin heavy chain is known to bind antibodies non-specifically (see, for example, Kirchausen *et al.*, 1983), so control experiments were also carried out, using at the same range of concentration (1 – 10 μg/ml), three, unrelated monoclonal IgMs and one monoclonal IgG prepared in this laboratory. In three cases there was no reaction with the clathrin heavy chain (Figure 1, lane 10). For this latter IgM, which is directed against the nuclear lamins (41CC4), a semi-quantitative comparison was carried out using an affinity-purified rabbit anti-mouse antibody and [^{125}I]protein A to localize the bound IgM. Monoclonal DC41 was able to detect the clathrin heavy chain at an antigen concentration 32-fold lower than that of 41CC4.

Comparable experiments using the affinity-purified polyclonal anti-clathrin gave the results presented in Figure 2. In contrast to the monoclonal antibody, it could detect clathrin in total extracts derived from WI38 cells (Figure 2, lanes 1,2) or rat liver (Figure 2, lanes 3,4) as well as in triskelions derived from rat liver (Figure 2, lanes 5,6) or pig brain (Figure 2, lanes 7,8). It could also detect clathrin at an antibody concentration

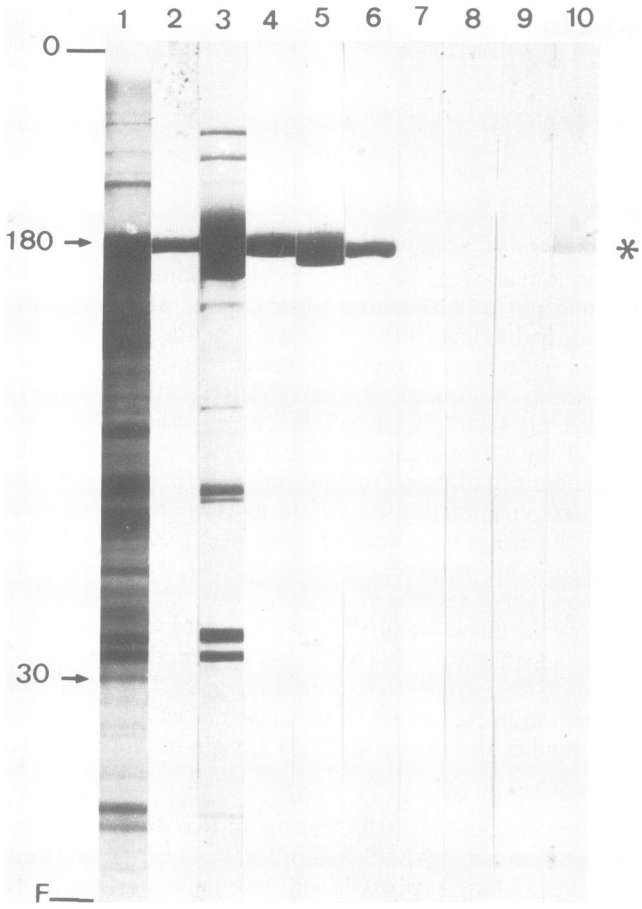


Fig. 1. Monoclonal antibody DC41. Crude coated vesicles (from the first sucrose gradient in the method of Pearse, 1975) from pig brain **(lanes 1,2)**, rat liver triskelions **(lanes 3,4)** and pig brain triskelions **(lanes 5–10)** were fractionated by SDS-PAGE and either stained using Coomassie blue **(lanes 1,3,5)** or immunoblotted using DC41 **(lanes 2,4,6)** or unrelated monoclonal antibodies against vimentin **(lane 7)**, a Golgi membrane protein **(lane 8**, Burke *et al.*, 1982), α-tubulin **(lane 9)** and lamin **(lane 10**, Burke *et al.*, 1983). The bound antibodies were visualized using a second antibody coupled to HRP followed by the peroxidase reaction. Note that DC41 only labelled the clathrin heavy chain, and, of the control monoclonal antibodies, only the anti-lamin antibody reacted weakly with the clathrin heavy chain (star). O is the origin and F the dye front.

10 times lower than that needed for DC41; as little as 1 ng of clathrin could be detected on the polyclonal antibody on nitrocellulose filter. This antibody also detected both the heavy and light chains of clathrin even though the latter have different mol. wts. depending on the source. In fact we have not been able to find a clathrin with which this antibody does not react. This is in contrast to polyclonal anti-clathrins prepared by other workers which tend only to recognize clathrin from certain sources (see for example, Kartenbeck *et al.*, 1981).

Immunofluorescence microscopy

WI38 cells were fixed, opened with Triton X-100, and

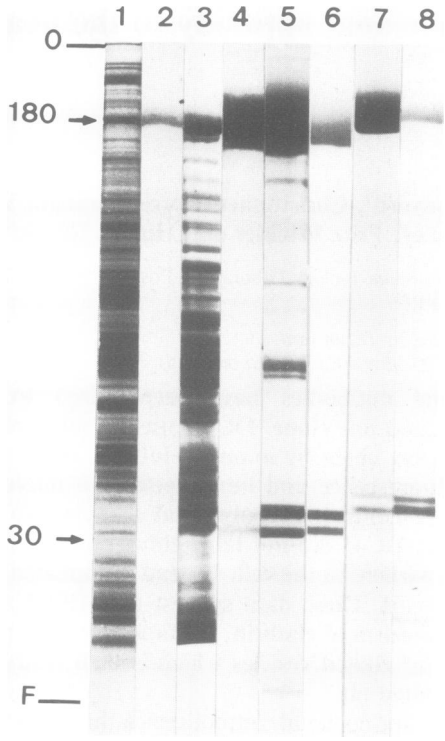
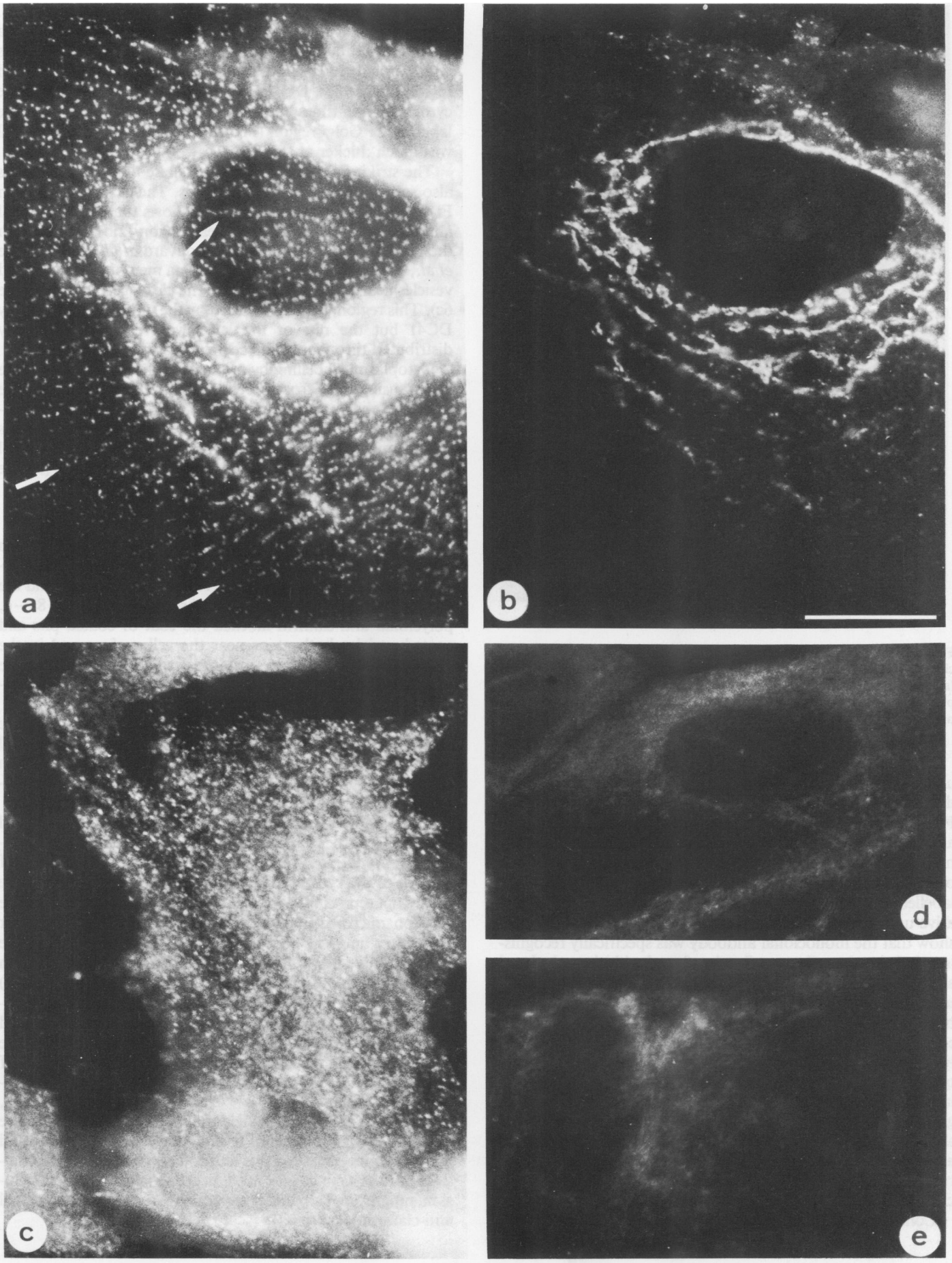


Fig. 2. Polyclonal antibodies to clathrin. Total extracts of WI38 cells **(lanes 1,2)** and rat liver **(lanes 3,4)**, rat liver triskelions **(lanes 5,6)** and pig brain triskelions **(lanes 7,8)** were fractionated by SDS-PAGE and either stained using Coomassie blue **(lanes 1,3,5,7)** or immunoblotted using polyclonal anti-clathrin **(lanes 2,4,6,8)** which was visualized using a second antibody bound to HRP followed by the peroxidase reaction. Note that the antibody detected both the heavy and light chains of clathrin. O is the origin and F the dye front.

labelled with polyclonal anti-clathrin which was visualized using a rhodamine-conjugated second antibody. Labelling was only seen once the cells had been opened with Triton X-100 and the punctate labelling of the plasma membrane presumably represented labelling of coated pits (Figure 3a). These were frequently aligned along stress fibers. By changing the plane of focus to the middle of the cell, it was possible to see labelling of a reticular network in a perinuclear region (Figure 3b) which appeared to coincide with the Golgi complex. This was supported by double-label experiments using fluorescein-conjugated WGA as a marker for the Golgi complex (Virtanen *et al.*, 1980) (cf., Figure 4a and b) but the precise location demanded electron microscopic studies which are described below.

The labelling pattern obtained using monoclonal antibody DC41 was completely different (Figure 3c and d). Again no labelling was found until the cells were opened with Triton X-100 and the labelling pattern was still punctate. The punctate pattern cannot be taken to imply a discrete structure. It is our experience that even soluble, cytoplasmic proteins give a punctate pattern when labelled with specific antibodies. We do not yet understand the reason. Instead of labelling just

Fig. 3. Immunofluorescence labelling of WI38 cells using either polyclonal anti-clathrin **(a,b)** or monoclonal DC41 **(c)**. Polyclonal anti-clathrin gave punctate labelling near the cell surface which appeared to follow stress fibres (arrows) **(a)** and, at a lower focal plane, labelling of a perinuclear reticulum which was the Golgi complex **(b**, see Figure 4). Monoclonal DC41 gave punctate labelling throughout the cell cytoplasm **(c)**. Cells were also pre-treated with polyclonal anti-clathrin before labelling with DC41 and a completely different pattern was obtained **(d)** showing that the polyclonal antibody blocked binding of DC41. The pattern was a diffuse cytoplasmic labelling wth occasional labelling of reticular and filamentous structures, and was identical to that obtained by labelling the cells with the rhodamine-conjugated second antibody alone **(e)**. **(d)** and **(e)** needed longer exposures than **(c)** to be visible in the same printing conditions. Magnification: x 1250, bar 20 μm.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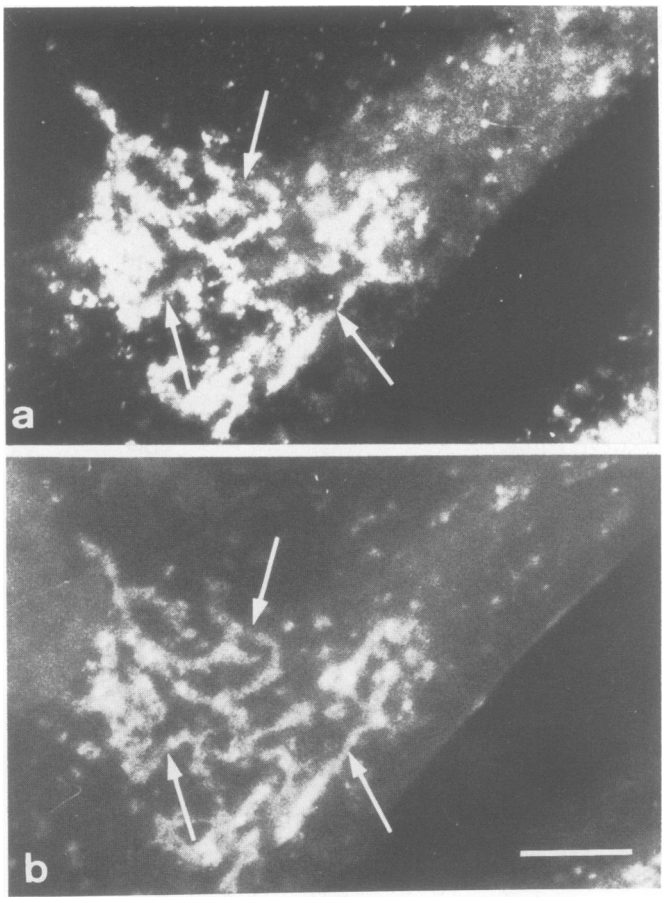


Fig. 4. Immunofluorescence labelling of WI38 cells using polyclonal anti-clathrin visualized using a rhodamine-conjugated sheep anti-rabbit antibody **(a)**, and fluorescein-conjugated WGA **(b)** as a marker for the Golgi complex (Virtanen *et al.*, 1980). With a few exceptions (arrows) the reticular patterns were almost identical. Magnification: x 1450, bar: 10 μm.

under the cell surface and in the Golgi region, the dots were distributed randomly throughout the cell cytoplasm (compare Figure 3a,b and c) with no labelling of the nuclear interior. In addition, the numbers of dots in the cytoplasm exceeded the number seen with the polyclonal anti-clathrin by at least one order of magnitude. Filaments and microtubules were never labelled and the pattern was not affected by the type of fixative used (formaldehyde, methanol-acetone or ethanol). To show that the monoclonal antibody was specifically recognising clathrin, the cells were first incubated with the polyclonal antibody at a concentration 10-fold higher than that normally used for labelling, to ensure saturation of all antibody-binding sites on clathrin. Labelling with DC41 then gave a completely different pattern (Figure 3d) and one which closely resembled that obtained by labelling the cells with the second antibody alone (Figure 3e). The converse experiment, labelling first with DC41 and then polyclonal anti-clathrin, gave the expected polyclonal antibody pattern (data not shown). Since the polyclonal antibody recognized clathrin alone in whole homogenates of WI38 cells (Figure 2), these results show that DC41 recognises a cytoplasmic form of clathrin that is also recognised by the polyclonal antibody.

Comparable experiments using DC41 were also carried out using NRK cells and similar results were obtained (Figure 5). The polyclonal anti-clathrin gave dots all over the plasma membrane (though this was not as clear as with WI38 cells which are much flatter) and preferential labelling of the Golgi region (Figure 5a). DC41 gave punctate labelling of the entire cytoplasm and though there appeared sometimes to be more label in the Golgi region (Figure 5c) this was because the cell was much thicker at this point.

The striking differences between these two antibodies was also seen in cells undergoing mitosis. The example shown in Figure 6, of a telophase NRK cell, shows that the polyclonal anti-clathrin labelled a perinuclear region known to contain the reassembled Golgi complex (Louvard *et al.*, 1982; Burke *et al.*, 1982), coated pits on the plasma membrane and coated vesicles near this membrane, and the cleavage furrow (Figure 6c). This region was also labelled by the monoclonal antibody DC41 but the rest of the label was relatively uniformly distributed throughout the cell cytoplasm (Figure 6a).

Cytoplasmic labelling by monoclonal DC41 was seen more clearly using thin, frozen sections of rat liver (Figure 7). The punctate labelling by the polyclonal antibody was mostly concentrated at the plasma membrane (Figure 7b), whereas monoclonal DC41 gave uniform labelling of the cell cytoplasm with no labelling of the nuclear interior (Figure 7a).

Immunoelectron microscopy

WI38 cells were fixed, opened with saponin, labelled with polyclonal anti-clathrin followed by a second antibody conjugated to HRP, the peroxidase reaction carried out, and the product localized using osmium. As shown in Figure 8a–c, polyclonal anti-clathrin labelled the coat of coated pits and coated vesicles both at or near the cell surface and in the Golgi region. In those instances where the coats were unlabelled, this was probably a consequence of poor penetration by the antibody into the cell.

Multi-vesicular bodies (MVB) are frequently found to have surface-dense plaques and there have been suggestions based on their morphology that they might contain clathrin (Holtzman, 1976). As shown in Figure 8d and e, these plaques were labelled by the polyclonal anti-clathrin antibody.

Comparable experiments using monoclonal DC41 proved difficult to do because the IgM is a large molecule and cross-linking conditions that allowed subsequent removal of enough of the cytosol to allow entry of this antibody into the cell cytoplasm resulted in poor preservation of structure at the electron microscopic level. Using borderline conditions for IgM entry and low concentrations of glutaraldehyde in the fixative, only a few cells out of every hundred were labelled in parallel immunofluorescence experiments (data not shown) but these cells were easily identified in the electron microscope. The label was not found to be concentrated in coated pits or coated vesicles (Figure 9a). Instead it was found in the cell cytoplasm and attached to the cytoplasmic surfaces of many subcellular structures, particularly the rough endoplasmic reticulum. Using 8% formaldehyde as the fixative, nearly all the cells were labelled though the very fine structure was very poorly preserved. It was, however, possible to find many profiles of coated pits and coated vesicles and in all instances they were clearly not labelled (Figure 9b,c) a conclusion supported by parallel experiments using the polyclonal anti-clathrin (Figure 9d).

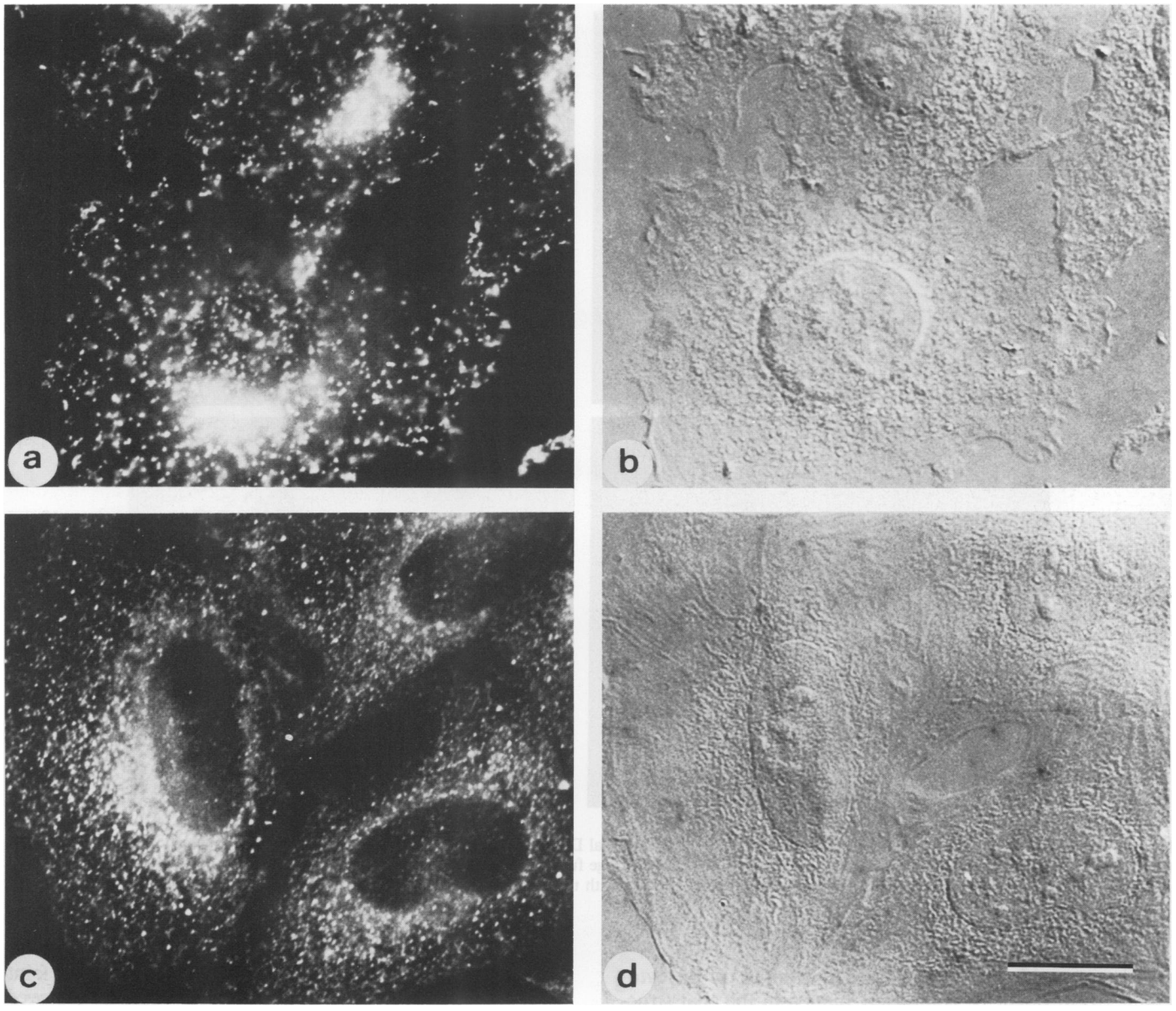


Fig. 5. Immunofluorescence labelling of NRK cells using monoclonal DC41 **(a,b)** or polyclonal anti-clathrin **(c,d)**. Polyclonal anti-clathrin labelled the cell surface and a perinuclear region **(c)** whereas DC41 labelled the entire cell cytoplasm **(a)**. The fluorescence pictures **(a,c)** are presented with the corresponding Nomarski pictures **(b,d)**. Magnification: x 1100, bar: 20 μm.

Discussion

We have isolated and characterized monoclonal DC41 which recognizes the heavy chain of clathrin bound to nitrocellulose filters, but not that in coated pits and coated vesicles, by immunofluorescence or immunoelectron microscopy. Instead, it labels discrete structures in the cell cytoplasm and the simplest interpretation would be to suggest that it recognizes a cytoplasmic form of clathrin. However, a number of technical problems must first be discussed.

In our experience, and that of others, both the clathrin heavy chain and IgM molecules are rather 'sticky'. It was important for us, therefore, to show that DC41 was binding specifically to clathrin in immunoblotting studies. We did this by testing three unrelated IgMs and an IgG for their ability to bind to the clathrin heavy chain. Three of them did not bind at all and the other one bound weakly when used at antigen concentrations at which DC41 bound strongly. The binding in this instance was determined semi-quantitatively and DC41 bound at a dilution 32 times greater than the dilution of this control IgM. DC41 also recognized only the clathrin heavy chain in crude preparations of coated vesicles.

Monoclonal DC41 labelled clathrin in the cell cytoplasm in immunofluorescence and immunoelectron microscopic studies. The labelling was specific because it was abolished by pre-incubation with the polyclonal antibody and it was punctate suggesting that the antibody might be binding to discrete structures in the cell cytoplasm. Labelling by DC41 does, however, beg the important question as to why the cytoplasmic labelling is not seen using the polyclonal anti-clathrin. It is relatively easy to understand why the monoclonal anti-

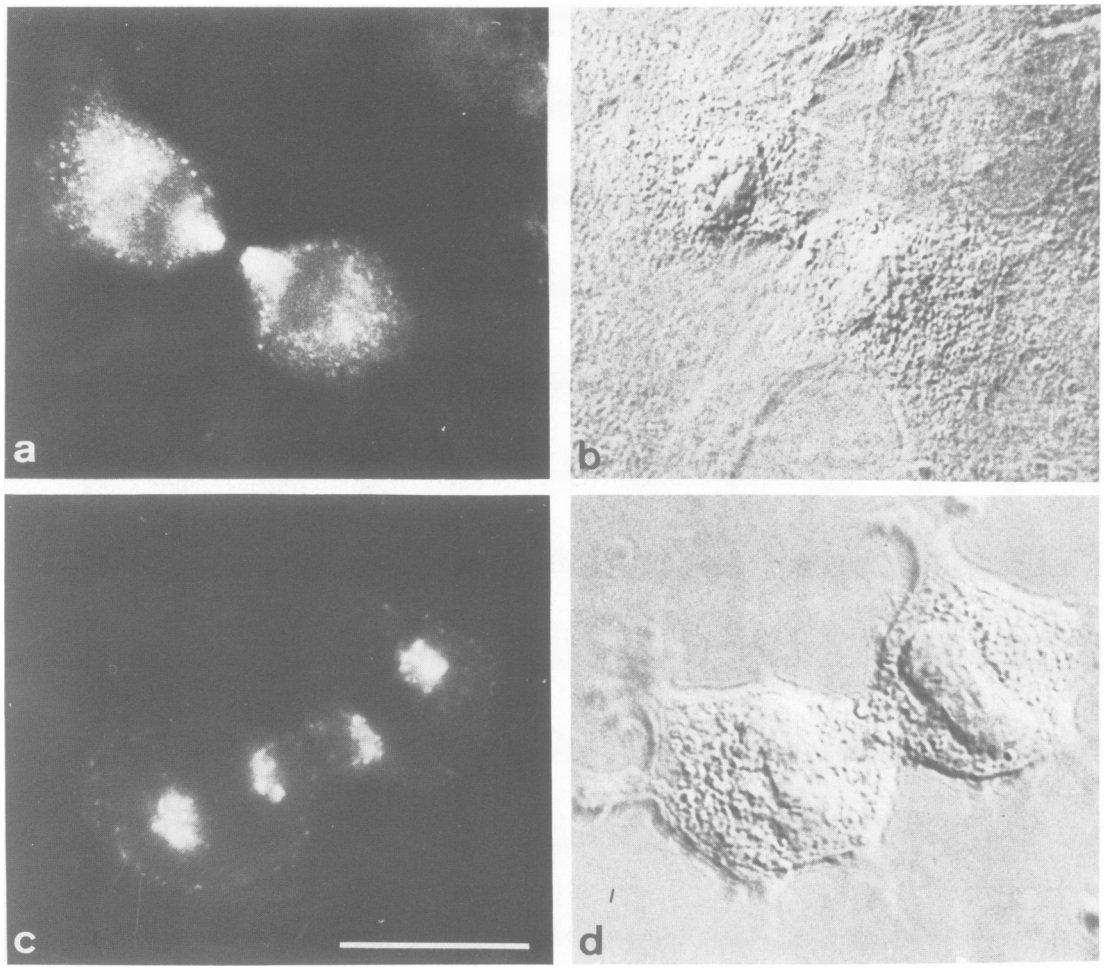


Fig. 6. Immunofluorescence labelling of a telophase NRK cell using monoclonal DC41 **(a,b)** or polyclonal anti-clathrin **(c,d)**. Monoclonal DC41 gave dots throughout the cell cytoplasm with a concentration in the region of the cleavage furrows **(b)**. Polyclonal anti-clathrin labelled both the cleavage furrow and a perinuclear region **(c)**. The fluorescence pictures **(a,c)** are presented together with the corresponding Nomarski pictures **(b,d)**. Magnification: x 1250, bar: 20 μm.

body is so specific because an epitope exposed on the cytoplasmic form might be completely hidden in the coat structure, but the polyclonal antibody recognizes epitopes on both forms as shown by the competition experiments (Figure 3). The explanation probably lies in the relative local concentrations of the different forms of clathrin in the cell and in the procedures normally adopted in immunofluorescence studies. Coated vesicles contain at least 36 triskelions, each of which have diameters comparable with that of the coated vesicles they comprise. The concentration of clathrin antigen in coated pits and coated vesicles is thus probably much higher than that in the cytoplasmic triskelion pool. Under these circumstances, the polyclonal antibody would only give faint labelling of the cytoplasm. This, in turn, would be removed because, in setting up immunofluorescence studies with a new antibody, it is usual to test a series of dilutions and use the one giving the highest signal to noise ratio. Cytoplasmic labelling is generally regarded as noise so the anti-clathrin antisera would be diluted until one could see bright dots (coated pits and coated vesicles) against a black background. It is of interest that we have found differences between polyclonal anti-clathrin raised in different rabbits and that one, in particular, gave a high cytoplasmic staining in addition to labelling of coated pits and coated vesicles, suggesting that it

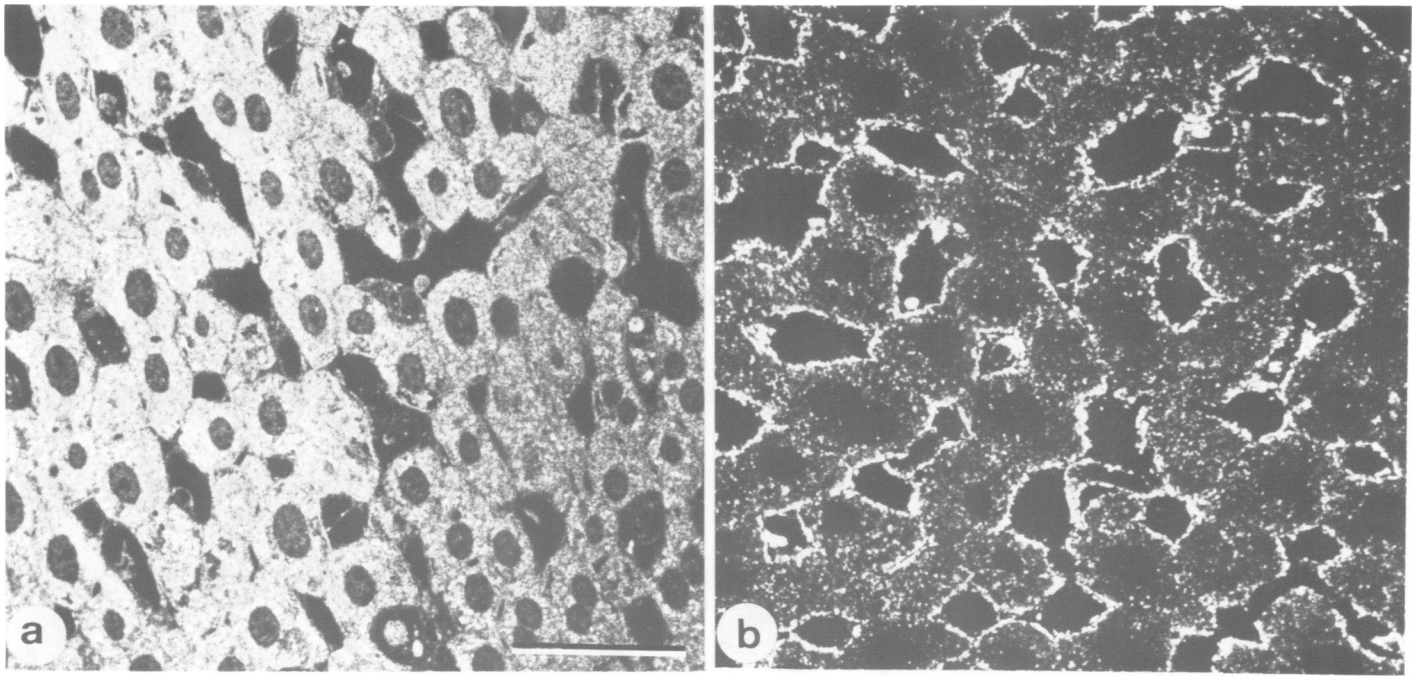


Fig. 7. Semi-thin (~0.5 μm) frozen sections of rat liver labelled with either monoclonal DC41 **(a)** or polyclonal anti-clathrin **(b)**. The polyclonal antibody gave a punctate labelling pattern especially at the cell surface with a higher concentration at the sinusoidal front, whereas DC41 labelled the entire cell cytoplasm but not the nuclear interior. Magnification: x 1150, bar: 20 μm.

recognized more epitopes on the cytoplasmic form of clathrin than the other polyclonal antisera (data not shown). This antibody was actually more efficient in competition experiments using DC41.

In summary, we suggest that monoclonal DC41 recognizes a cytoplasmic form of clathrin which might be triskelions resulting from coated vesicle uncoating and on their way to rebuild more coated pits. We are now trying to confirm this suggestion by microinjecting the antibody in an attempt to disrupt cellular processes known to be mediated by clathrin.

Materials and methods

Clathrin for immunisation

Polyclonal antibodies were raised to empty clathrin cages prepared from pig brain coated vesicles (Pearse, 1975) by extraction with 2 M urea which was subsequently removed by dialysis (Blitz *et al.*, 1977). To improve their antigenicity, these cages were coupled to keyhole limpet hemocyamin (KLH) using the procedure of Ternynck and Avrameas (1976a) with the following modifications designed to keep the activated KLH in solution. KLH (20 mg) was dissolved in 2 ml H_2O and 0.1 ml 3 M NaCl and 0.2 ml 1 M phosphate buffer, pH 7.4 were then added. P-benzoquinone (BQ, 15 mg, 3 x recrystallized) in 0.6 ml ethanol was then added and the mixture incubated for 15 min in the dark at room temperature. Excess BQ was removed from the resulting activated KLH by Sephadex G50 (fine) chromatography and 4 mg of this conjugate was mixed with 1 mg of clathrin in 0.2 M Na_2CO_3-$NaHCO_3$ buffer pH 9.0 and incubated at room temperature for 16 h in the dark. A small precipitate was sometimes formed which was easily removed by a 5 min centrifugation in an Eppendorf microfuge. The mixture was concentrated by dialysis against 50% (v/v) glycerol in phosphate-buffered saline (PBS) at 4°C. This was stored for up to 1 year at −20°C. The coupling efficiency was determined by running samples of clathrin, before and after conjugation, on SDS-polyacrylamide gels and the percentage that still had a mol. wt. of 180 K was estimated. We usually found that 60−70% of the clathrin was coupled to KLH.

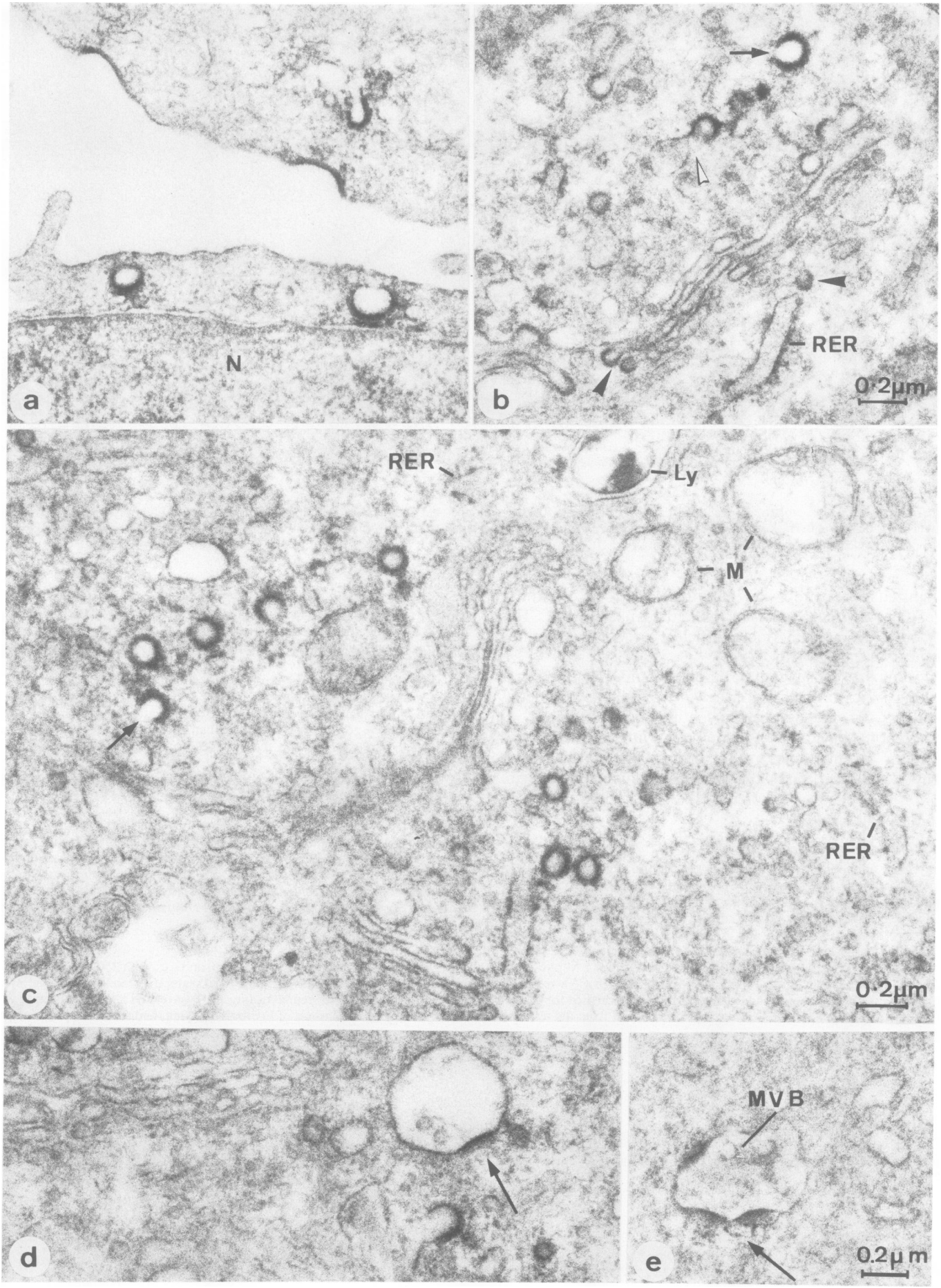
Monoclonal antibodies were raised against pig brain triskelions prepared as described by Winkler and Stanley (1983) and conjugated to KLH.

Polyclonal antibodies

Rabbits were immunized with KLH-clathrin using an immunisation regime described previously (Louvard *et al.*, 1982). The serum had a low titre of anti-clathrin antibodies but these were of high affinity. The serum was affinity-purified on columns containing either triskelions coupled to acrylamide-agarose beads (Ternynck and Avrameas, 1976b) or coated vesicles coupled to HMD-Ultrogel beads (Coudrier *et al.*, 1981).

Monoclonal antibodies

Two mice were each injected, both i.p. and s.c., with 350 μg KLH-clathrin in complete Freund's adjuvant. Four weeks later they received a similar injection but in incomplete Freund's adjuvant. Twelve days later this injection was repeated using PBS instead of Freund's adjuvant, and then 11 days after that,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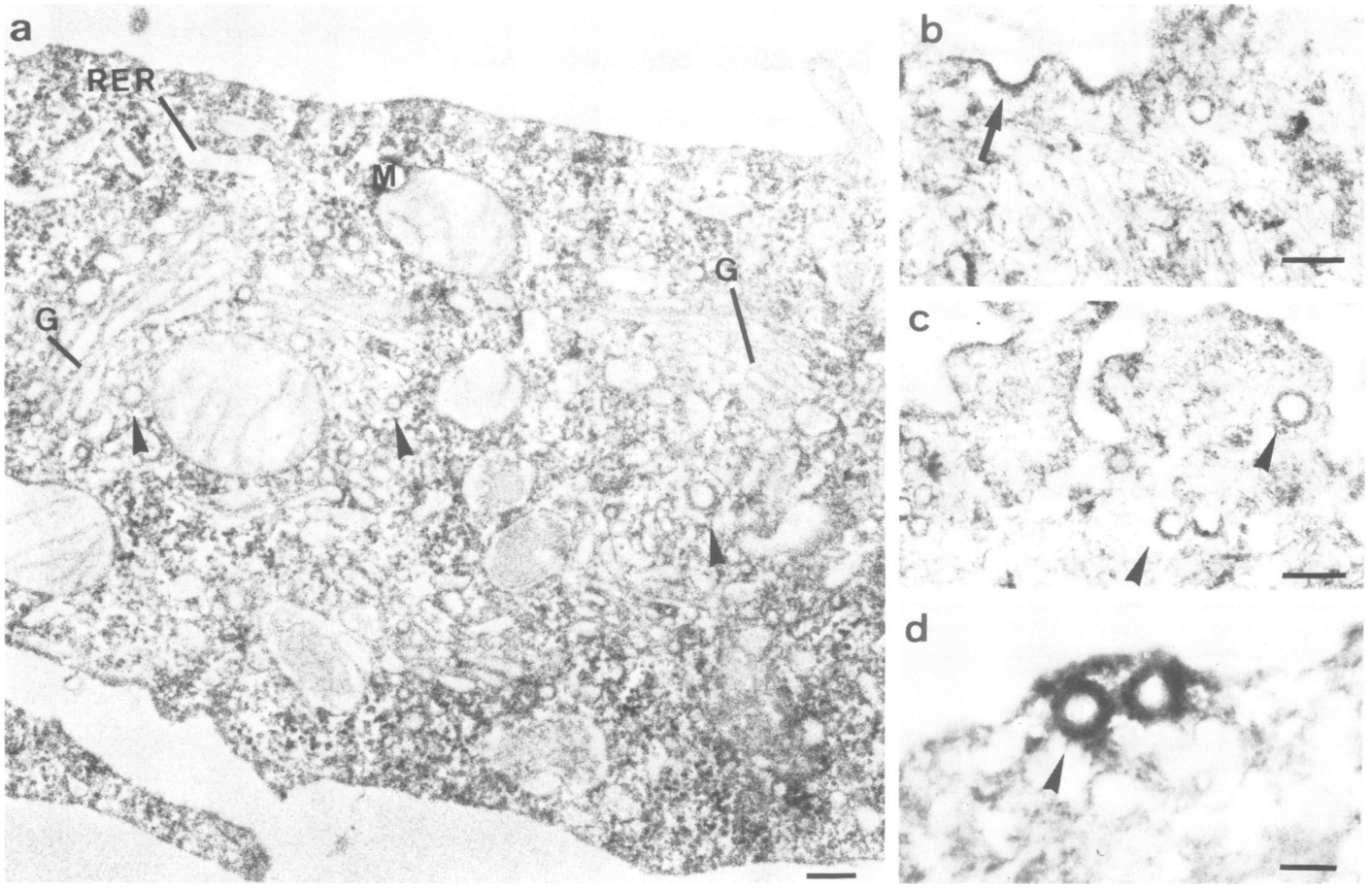

Fig. 9. Immunoperoxidase labelling of WI38 cells using monoclonal DC41. **(a)** Samples fixed in 0.01% glutaraldehyde and 2% formaldehyde. Preservation was sufficient to identify most of the cellular organelles and coated vesicles (arrowheads) were not labelled. The label was found in the cell cytoplasm and attached to the cytoplasmic surface of organelles particularly in the rough endoplasmic reticulum (RER). **(b,c)** Samples fixed in 8% formaldehyde. The morphological preservation was very poor but coated pits (arrows) and coated vesicles (arrowheads) were easily identified and were not labelled. **(d)** As for **b,c** but labelled with polyclonal anti-clathrin as a positive control for labelling of clathrin-coated structures (arrowhead). M, mitochondria; G, Golgi. Magnification: **a** x 34 000, **b,c** and **d** x 42 000, bar: 0.2 μm.

120 μg of KLH-clathrin in PBS was injected i.m. on each of three successive days. On the last of these three days about one quarter of the antigen was injected into the tail vein. Two days later spleen cells were removed and the cells dissociated and fused with SP2/O-Ag14 myeloma line (Schulman *et al.*, 1978) according to procedures described by Galfre *et al.* (1977). After fusion, the cells were gently suspended in Dulbecco's minimal essential medium (MEM) containing 10% (v/v) foetal calf serum (FCS) and 10% (v/v) new born calf serum, penicillin/streptomycin, hypoxanthine, aminopterin and thymidine, and distributed into 5 x 24 well tissue culture trays. After 2–3 weeks, supernatants from cultures positive for hybrid cell growth were screened for specific antibodies either by immunofluorescence microscopy as described previously (Burke *et al.*, 1982) or by immunoradiometric assay as follows.

Pig brain triskelions (1 μg) were added to each well of a 96 place microtitre plate (Dynatech, Alexandrinia, VA) coated with polylysine. After 1 h at room temperature, unbound triskelions were washed away using distilled water and any remaining binding site on the plastic were quenched by a 15 min treatment with 0.5% (w/v) gelatin in PBS. Culture supernatants (100 μl) diluted 1:10 in 100 mM phosphate pH 7.4 and containing 10 mg bovine serum albumin and 1 μg goat IgG/ml were added to the wells and incubated for 16 h at 20°C. Unbound material was washed away using deionised water. ^{125}I-labelled sheep anti-(mouse immunoglobulin) (7000 c.p.m.) was then added to each well and the plate incubated for a further 24 h at 20°C. The plates were then washed and the wells counted in a gamma counter. Out of 120 culture supernatants, 24 were positive, having 3–8 times the level of background radioactivity. After further screening by indirect immunofluorescence on NRK cells (see below) the hybridomas were cloned in agarose (Indubiose, Industrie Biologique Francaise; Coffino and Scharff, 1971) and injected into pristane-primed mice to form ascites tumours (Koprowski *et al.*, 1977).

Immunoblotting

This was carried out as described by Burnette (1981) with the modifications described previously (Burke *et al.*, 1982). The polyclonal anti-clathrin could detect as little as 10 ng of clathrin on the nitrocellulose filter whereas the monoclonal antibody required 100 ng of the heavy chain when using [^{125}I]protein A. The amount detected was 10 times lower using peroxidase-Fab.

Fig. 8. Immunoperoxidase labelling of WI38 cells using polyclonal anti-clathrin. There was specific labelling of coated pits and coated vesicles near the cell surface **(a)** and in the Golgi region **(b,c)**. In **(b)** there were a number of vesicles (arrow heads) that appeared to be budding from Golgi cisternae. Very occasionally, vesicles were seen to bud from what appeared to be smooth ER in the Golgi region (white arrowhead). **(c)** is an overall view of the Golgi region showing labelled coated vesicles on both sides of the stack. In both **(b)** and **(c)** some vesicles appeared open (arrows), presumably a consequence of the action of saponin. The dense plaques on lysosomes and multi-vesicular bodies are thought to contain clathrin (see text) and as shown in **(d,e)** these plaques were labelled by the polyclonal antibody (long arrow). N, nucleus; M, mitochondria; Ly, secondary lysosome; RER, rough endoplasmic reticulum; MVB, multivesicular body. Magnification **a,b,d** and **e** x 45 000; **c** x 51 000, bar: 0.2 μm.

Cells

NRK cells were grown in MEM containing 10% (v/v) FCS. WI38 cells were grown in Dulbecco's MEM containing 10% (v/v) FCS. Both lines were grown at 37°C in a humidified CO_2 incubator (5% CO_2, 95% air).

Immunofluorescence microscopy

This was carried out on whole cells (Wang *et al.*, 1982) or thin, frozen sections (Tokuyasu, 1973).

Immunoelectron microscopy

Immunoperoxidase labelling was carried out as described by Ohtsuki *et al.* (1978) and Tougard *et al.* (1980) with the modifications described by Reggio *et al.* (1983). We found that for WI38 cells and the polyclonal anti-clathrin, the optimal fixation conditions were 2% formaldehyde, 0.03% glutaraldehyde. For monoclonal DC41 we used either 0.01% glutaraldehyde and 2% formaldehyde or a stepwise fixation up to 8% formaldehyde. The steps comprised 15 min at 2% formaldehyde and 10 min at 4%, 6% and 8%.

Reagents

Glutaraldehyde was obtained from Ladd Research Industries, Burlington, USA; diaminobenzidine hydrochloride from Sigma; sheep Fab anti-rabbit IgG conjugated to HRP from Institut Pasteur, Paris, France; [^{125}I]protein A from New England Nuclear.

Acknowledgements

We thank Paul Webster, Brigitte d'Arcy and Jennifer Siegler for excellent technical assistance; Brian Burke and Kai Simons for critical reading of the manuscript; and Annie Steiner for typing.

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