STRUCTURAL HETEROGENEITY OF ENDOCYTIC MEMBRANES IN MACROPHAGES AS REVEALED BY THE CHOLESTEROL PROBE, FILIPIN

ROBERTO MONTESANO1, PIERRE VASSALLI1 AND LELIO ORCI1

1 Institute of Histology and Embryology, and 2 Department of Pathology, University of Geneva, Geneva, Switzerland

SUMMARY

The polyene antibiotic, filipin, by specifically interacting with cholesterol, produces ~25-nm protuberances (filipin-sterol complexes) in freeze-fractured membranes, and the addition of filipin to aldehyde fixatives has been recently introduced as a cytochemical technique for the localization of cholesterol in cell membranes. In a previous study we showed that, in fibroblasts, filipin-sterol complexes are absent from endocytic coated pits. To establish whether the absence of filipin-sterol complexes is a phenomenon restricted to coated pits or is correlated with endocytosis in general, we applied the filipin probe to cultured mouse peritoneal macrophages, in which different forms of endocytosis take place. The macrophages were incubated with bovine albumin or concanavalin A (Con A) to induce pinocytosis, and with heat-killed staphylococci or opsonized erythrocytes to induce phagocytosis, then fixed in glutaraldehyde/filipin and freeze-fractured. Filipin—sterol complexes were plentiful on the plasma membrane, on the smooth-membrane invaginations and vesicles induced by albumin, on the large endocytic vacuoles induced by Con A, and on the membrane of phagosomes but, in contrast, they were absent from coated pits and vesicles, as well as from coated segments of invaginations or vesicles. These results indicate that the membranes involved in different types of endocytosis do not react in the same way with filipin and may, therefore, have a different cholesterol content. This could reflect different mechanisms of formation for the various types of endocytic vesicles.

INTRODUCTION

During endocytosis, segments of the plasma membrane are invaginated into the cytoplasm and are subsequently pinched off from the surface to form intracellular vesicles (Silverstein, Steinman & Cohn, 1977). Whether the membrane of the nascent endocytic vesicle has the same composition as the plasma membrane from which it arises or, in contrast, shows relative enrichments and depletions of specific components is still a matter of debate (Tsen & Berlin, 1971; Ukena & Berlin, 1972; Oliver, Ukena & Berlin, 1974; Berlin & Fera, 1977; Straus, Imhoff & Bonventre, 1977; Silverstein et al. 1977; Edelson & Cohn, 1978; Orci et al. 1978; De Bruyn, Michelson & Becker 1978; Charalampous, 1979; Goldstein, Anderson & Brown, 1979; Muller, Steinman & Cohn, 1980; a, b; Mellman, Steinman, Unkeless & Cohn, 1980; Smolen & Karnovsky, 1980a, b; Cramer, Bainton & Werb, 1980; Nowakowski & Bianco, 1980). In a previous paper (Montesano, Perrelet, Vassalli & Orci, 1979) we used the cytochemical probe, filipin (Elias, Friend & Goerke, 1979; Robinson & Karnovsky, 1980a), to study the distribution of a major membrane lipid, cholesterol, at the level of the plasma
membrane and of endocytic invaginations in freeze-fractured fibroblasts. Filipin-sterol complexes, recognizable as ~ 25-nm protuberances scattered on the membrane faces (Verkleij et al. 1973; Tillack & Kinsky, 1973; Kitajima, Takashi & Nozawa, 1976; Andrews & Cohen, 1979; Montesano, 1979a, b; Elias et al. 1979; Robinson & Karnovsky, 1980a, b; Orci, Montesano & Brown, 1980; Montesano, Vassalli, Perrelet & Orci, 1980; Orci et al. 1980a, 1981; Nakajima & Bridgman, 1981), were absent from bristle-coated invaginations, suggesting that the latter have a lower cholesterol content than the rest of the plasma membrane (Montesano et al. 1979). Coated invaginations or pits (Roth & Porter, 1964; Friend & Farquhar, 1967; Anderson, Goldstein & Brown, 1976; Orci et al. 1978; Heuser, 1980) are being increasingly involved in a specialized form of endocytosis, which has been referred to as adsorptive or receptor-mediated endocytosis (Goldstein, et al. 1979; Brown & Goldstein, 1979). Therefore, our observations raised the question of whether the absence of filipin-sterol complexes is a phenomenon restricted to coated pits or is shared by other forms of endocytosis. To explore this issue we used the peritoneal macrophage as a model system, since in this cell type different forms of endocytosis can be induced experimentally (Cohn, 1970; Allison & Davies, 1974a, b; Zuckerman & Douglas, 1979). Our results indicate that, among the membranes involved in different types of endocytosis, only those appearing coated, whether corresponding to vesicles or invaginations, are not labelled by filipin*.

MATERIALS AND METHODS

Collection and cultivation of macrophages

Stimulated peritoneal macrophages were collected from C57 mice injected intraperitoneally 3 days previously with 2 ml of 3 % thioglycollate broth (Difco, Detroit, Mich.). They were washed, resuspended in Dulbecco's modified Eagles' medium (DMEM) supplemented with 5 % foetal calf serum (FCS) and allowed to attach (2 h, 37 °C) on Falcon Petri dishes (Falcon Plastics, Div. of Bioquest., Oxnard, Calif.) at 2 x 10⁵ cells/cm² (Cohn & Benson, 1965). After the phase of cell attachment, non-adherent cells were removed by repeated washings with DMEM.

Induction of endocytosis

To stimulate pinocytosis, macrophages were incubated in DMEM + 30 % FCS for 24 h at 37 °C, washed twice with DMEM and reincubated in DMEM + 1 % FCS + 40 mg/ml bovine serum albumin (BSA) (Pentex Path-o-cyte 5, Miles) for 150 min (Cohn & Parks, 1967). Control macrophages were incubated in DMEM + 50 % FCS for 24 h, washed and reincubated in DMEM + 1 % FCS for 150 min. To induce the formation of large endocytic vacuoles, macrophages were incubated in DMEM + 5 % FCS containing 50 μg/ml concanavalin A (Con A) (Pharmacia Fine Chemicals) for 2 h (Edelson & Cohn, 1974; Goldman, 1974; Goldman & Raz, 1975).

To induce phagocytosis, macrophages were incubated with either formalin-fixed and heat-killed bacteria (Staphylococcus aureus) prepared according to Kessler (1975) for 5 min to 2 h, or for 15 min with sheep erythrocytes, previously opsonized by coating them with rabbit anti-sheep-erythrocyte antibodies at a non-agglutinating dilution.

* Preliminary reports of this work were presented at the 12th Annual Meet. of Swiss Soc. Exp. Biol. (Basle, March 13–14, 1980), and at the 2nd Int. Congr. Cell Biol. (Berlin, August 31–September 5, 1980) and have appeared in abstract form (Montesano, Vassalli & Orci, 1980a, b.)
Processing for electron microscopy

At the end of each experiment, the macrophages were washed with DMEM and fixed for 30 min in 2.5% glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4. The cells were then gently scraped with a rubber policeman, pelleted, and resuspended in the same fixative containing 300 μM-filipin (Upjohn) (Elias et al. 1979) for at least 2 h, at room temperature. The glutaraldehyde/filipin solution was prepared by dissolving filipin in a small volume of dimethyl sulphoxide (DMSO), which was then added to the fixative to obtain the desired concentration. The final solution contained 1% DMSO.

For freeze-fracturing, filipin-treated macrophages were pelleted, cryoprotected with 30% glycerol in cacodylate buffer, mounted on gold discs and quickly frozen in the liquid phase of partially solidified Freon 22. Specimens were fractured and shadowed in Balzers BAF300 and BAF301 devices at a stage temperature of —110 °C. Replicas were cleaned by sequential treatment with sodium hypochlorite and chloroform/methanol and recovered on coated 150-mesh copper grids. For thin-section electron microscopy, the macrophages were processed as described previously (Montesano et al. 1979). Both freeze-fracture replicas and thin sections were examined in a Philips EM300 electron microscope.

RESULTS

Pinocytosis

In freeze-fracture replicas, the plasma membranes of filipin-treated macrophages are heavily labelled with filipin-sterol complexes, which are identified either as ~ 25-nm protuberances or slightly smaller pits (see Introduction for references) (Fig. 1). In addition, filipin-sterol complexes are also present on the membrane of large surface invaginations and cytoplasmic vesicles (Fig. 1). Similar findings are obtained in macrophages whose pinocytic activity was enhanced by incubating them in the presence of BSA (40 mg/ml) (Cohn & Parks, 1967); in these conditions a heavy filipin labelling is always observed on complex invaginations of the cell surface and on endocytic vesicles and vacuoles (Fig. 2). However, in both control and BSA-stimulated macrophages, filipin-sterol complexes are consistently absent from another type of surface invagination. The latter appear as circular depressions of rather uniform size closely resembling the coated pits previously described in fibroblasts (Montesano et al. 1979) (Fig. 3). However, reliable identification of these depressions as coated pits is possible only in thin-section electron microscopy. In thin sections of dehydrated and embedded material, the formation of filipin-sterol complexes results in a wavy appearance of cell membranes with frequent loss of their trilaminar structure (Elias et al. 1979; Montesano et al. 1979; Robinson & Karnovsky, 1980a). In macrophages, this alteration is evident at the level of the plasma membrane and of smooth-surfaced cytoplasmic vacuoles (Fig. 4). In striking contrast, the membrane of bristle-coated pits and vesicles appears unaffected by treatment with filipin and their trilaminar structure is well preserved (Fig. 4). Examination of thin sections thus confirms that the invaginations lacking filipin-sterol complexes in freeze-fracture replicas do correspond to bristle-coated pits. Sometimes, in freeze-fractured material, a non-labelled segment of membrane appears to be budding off from an otherwise heavily labelled surface invagination (Fig. 5). In this case again, examination of thin sections indicates that non-labelled membrane segments most likely correspond to the coated extremity of an invagination or of a vesicle (Fig. 6).
Filipin-sterol complexes, which appear as 25-nm protuberances (black circles) or slightly smaller pits (white circles) on membrane faces, are densely distributed on the plasma membrane, on irregularly-shaped membrane invaginations (large arrows), and on presumptive cytoplasmic vesicles (v). In cross-fractures, the membranes affected by the filipin treatment show a characteristic scalloped appearance (thin arrows). ES, extracellular space; pm, plasma membrane; cyt, cytoplasm. In all figures the encircled arrow indicates the direction of shadowing. X 32000.

Con A-induced vacuolization

Incubation of peritoneal macrophages with Con A (50 µg/ml) for 2 h results in the formation of numerous large, electron-lucent endocytic vacuoles within the cell cytoplasm (Edelson & Cohn, 1974; Goldman, 1974; Goldman & Raz, 1975) (Fig. 7, inset). When these macrophages are exposed to filipin and freeze-fractured, the limiting membrane of Con A-induced vacuoles appears heavily labelled with filipin-sterol complexes (Fig. 7).
Heterogeneity of endocytic membranes

Fig. 2. Freeze-fracture replica of a BSA-stimulated macrophage after filipin treatment. The peripheral cytoplasm presents a complex system of tubulo-vesicular structures of presumptive endocytic nature (in this fracture it cannot be established whether they are still surface-connected or not). The membrane of these structures appears heavily and uniformly labelled with filipin-sterol complexes. Notice that the latter appear here predominantly as protuberances on protoplasmic or P fracture-faces (Branton et al. 1975) and pits on E-faces. ES, extracellular space; pm, plasma membrane; cyt, cytoplasm. × 43,500.

Phagocytosis

Following incubation of peritoneal macrophages with suspensions of formalin-fixed and heat-killed staphylococci, numerous bacteria are seen to be contained within phagocytic vacuoles in the cell cytoplasm (Fig. 8). In freeze-fracture replicas of filipin-treated macrophages, phagocytic vacuoles are identified by their characteristic size and shape and by the fact that the fracture plane frequently steps from their
Fig. 3. En face view of the plasma membrane (P fracture-face) of a BSA-stimulated macrophage following filipin treatment. The plasma membrane (pm) is evenly studded with filipin-sterol complexes (circles) except at the level of shallow circular depressions (asterisks) closely resembling the coated pits previously described in fibroblasts (Orci et al. 1978; Montesano et al. 1979). × 69,000.

Fig. 4. Thin section of a peritoneal macrophage treated with filipin. The plasma membrane (pm) and the limiting membrane of 2 cytoplasmic vesicles (arrows), have lost their trilaminar structure and appear corrugated and ill-defined due to the formation of filipin–sterol complexes. In contrast, the membrane of a bristle-coated vesicle (cv) appears well preserved. × 59,500.

Fig. 5. Detail from a freeze-fracture replica of a filipin-treated peritoneal macrophage. A segment of membrane devoid of filipin–sterol complexes (asterisk) seems to bud off from an otherwise heavily labelled membrane invagination. ES, extracellular space; pm, plasma membrane. × 58,000.

Fig. 6. Detail from a thin section of a filipin-treated peritoneal macrophage. At one end of an endocytic vacuole (or invagination), whose limiting membrane is markedly altered by filipin, one sees a coated pit (cp), which is probably in the process of pinching off to form a coated vesicle. The trilaminar appearance of the membrane is preserved only at the level of its coated segment, indicating that the latter failed to react with filipin. × 57,000.
Fig. 7. Freeze-fracture replica of a macrophage incubated with Con A for 2 h and then treated with filipin. Following incubation with Con A, several large endocytic vacuoles (va) have formed in the cell cytoplasm (see inset for thin-section electron microscopy). When the membrane of these vacuoles is exposed by the fracture process, it appears heavily labelled with filipin-sterol complexes. × 37,500; inset, × 5,500.

limiting membrane to the richly particulate bacterial plasma membrane (Morioka, 1976) (Figs. 9, 10). In marked contrast with the absence of filipin-sterol complexes on the bacterial membrane (which does not contain cholesterol; for a review see Gel'man, Lukoyanova & Ostrovskii, 1975), the phagosomal membrane shows heavy labelling with filipin (Figs. 9, 10). Although phagocytic vacuoles are more readily identifiable after longer times of incubation with the bacterial suspension (due to the large number of engulfed staphylococci), those vacuoles observed at earlier times (5 min) also appear richly labelled with filipin-sterol complexes. This is also true of phagocytic vacuoles of macrophages that have engulfed erythrocytes (Fig. 11).

DISCUSSION

The results of the present experiments on mouse peritoneal macrophages indicate that the membranes involved in different types of endocytosis do not react in the same
Fig. 8. Thin section of a macrophage incubated for 2 h with a suspension of heat-killed bacteria (S. aureus). Numerous staphylococci (s) are contained within phagocytic vacuoles in the cell cytoplasm. n, nucleus. ×8000.

Figs. 9, 10. Freeze-fracture replicas of macrophages incubated for 2 h with a suspension of staphylococci. Several phagocytic vacuoles (pv), whose limiting membranes are richly labelled with filipin-sterol complexes, are visible in the cytoplasm. In some instances, the fracture plane jumped from the phagosomal membrane across the bacterial cell wall exposing the particle-rich plasma membrane (pm) of an engulfed staphylococcus. Notice the absence of filipin-sterol complexes on the bacterial membrane. es, extracellular space; pm, plasma membrane. Fig. 9, ×54000; Fig. 10, ×58000.
Heterogeneity of endocytic membranes

Fig. 11. Freeze-fracture replica of a macrophage incubated for 15 min with opsonized sheep erythrocytes. Both the plasma membrane of the macrophage (pm) and the limiting membrane of a large phagocytic vacuole (pv) show heavy labelling with filipin x 21,000.

way with the cholesterol probe, filipin. Filipin labelling was absent from the membrane of bristle-coated pits and vesicles, as well as from the coated segments of membrane invaginations or vacuoles. In contrast, all other forms of membranes related to endocytosis, i.e. non-coated pinocytic membrane invaginations and vesicles, Con A-induced pinocytic vacuoles and phagosomal membranes, were labelled with filipin to an extent comparable to that of the plasma membrane. Since filipin is a specific marker for membrane cholesterol (Norman, Spielvogel & Wong, 1976), these observations suggest a striking heterogeneity of endocytic membranes with regard to their cholesterol content. One could argue that the presence of the thick bristle-coat might somehow hinder the formation of filipin–sterol complexes, so that the lack of
reactivity of coated pits to filipin might not correspond to a greatly decreased cholesterol content. However, biochemical analysis done on isolated coated vesicles also indicates a markedly low cholesterol to phospholipid ratio in their membranes (Pearse, 1976). Furthermore, we have reported that filipin–cholesterol complexes were also absent from non-coated, flask-shaped micro-invaginations in fibroblasts (Montesano et al. 1979) (similar invaginations were not observed in the present study on macrophages). It is on the basis of these observations that we have suggested that cholesterol depletion, by producing a localized increase in membrane fluidity, could play a role in the process of endocytosis. This hypothesis led us to study macrophages, since these cells have very high rates of endocytosis and membrane internalization (Steinman, Brodie & Cohn, 1976), especially during increased pinocytic activity, following exposure to BSA at high concentration, to Con A, or following massive phagocytosis. The possibility suggested in our previous study seems to be contradicted by the results obtained with macrophages in these various conditions, since, in spite of the extent of endocytosis, the cholesterol content of the various membranes resulting from massive or on-going endocytosis was always high, except in the case of coated membranes. Thus, it now seems clear from the present observations with macrophages that cholesterol depletion is not a general correlate of membranes undergoing endocytosis or very recently internalized. However, this does not necessarily rule out the possibility that a localized increase in membrane fluidity due to a low sterol content could be of importance in some forms of endocytosis, such as that mediated by the coated pit/coated vesicle mechanism. Since it has been reported that modification of the sterol content of the plasma membrane profoundly affects endocytosis (Dianzani et al. 1975; Heiniger, Kandutsch & Chen, 1976; Heiniger & Marshall, 1979), it is conceivable that the differences in cholesterol levels between endocytic membranes could reflect different mechanisms of formation for the various types of vesicles.

In conclusion, although the exact significance of these observations cannot be appraised precisely at the present time, since the function of the clathrin coat is not yet clear, they reveal that the striking heterogeneity in the organization of the membranes involved in different types of endocytosis, shown by the existence of coated and non-coated membranes, is paralleled by marked differences in the cholesterol content of these membranes.

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