Interaction of plasma membrane-associated filaments and H-2 histocompatibility antigens before and after induced patching and capping

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Summary

The interaction of H-2 antigens and plasma membrane-associated filaments was studied on dry-cleaved preparations of immunogold-labelled lymphoma cells. In prefixed cells, the plasma membrane-associated network was isotropic without any prevailing direction of the filaments, and the gold-labelled H-2 antigens were preferentially localized over or at a very short distance from membrane-associated filaments. Incubation of unfixed cells with anti-H-2 antibodies followed by fixation and incubation with anti-Ig, did not induce detectable redistribution of H-2 antigens or of the filament network. Notwithstanding this apparent absence of rearrangement of H-2 antigens and filaments, a detergent-resistant linkage to the cytoskeleton was induced. Before immune incubations, virtually all H-2 antigens were solubilized by extraction with Triton X-100, while after incubation with anti-H-2 antibodies about 50 % of the H-2 antigens were linked to the Triton X-100-insoluble cytoskeleton. Sequential addition of anti-H-2 and anti-Ig antibodies to unfixed cells induced formation of patches and caps of H-2 antigens. Under these conditions, the majority of the H-2 antigens became linked to the detergent-resistant cytoskeleton. Redistribution into patches and caps was often accompanied by a local rearrangement of the isotropic network into bundles of parallel filaments immediately adjacent to the plasma membrane. Patches were seen to overly both isotropic networks and these parallel filaments. Large sheets of plasma membrane overlying parallel filaments were frequently devoid of gold-labelled H-2 antigens and coated pits, and thus most probably represented areas away from caps. This observation suggests that capping is accompanied by a rearrangement of filaments close to the membrane.

Key words: cytoskeleton, H-2 antigens, antigen redistribution, immunoelectron microscopy.

Introduction

Crosslinking of cell-surface molecules by multivalent ligands like lectins and immunoglobulins may lead to their aggregation into patches. Such patches can, either spontaneously or after further crosslinking by a second ligand, assemble into a cap at one pole of the cell. Several models have been proposed to explain the capping phenomenon (Bourguignon & Singer, 1977; De Petris, 1978; Klausner *et al.* 1980; Oliver & Berlin, 1982; Bourguignon & Bourguignon, 1984). In 1971 the involvement of contractile microfilaments was described by Taylor *et al.* (1971). In most models a direct or indirect linkage of the surface molecules with the cytoskeleton is supposed to be essential, while all models comprise a local reorganization of the cortical part of the cytoskeleton localized close to the plasma membrane.

We have shown that a spatial relation between cell surface molecules and filaments immediately adjacent to the inner face of the plasma membrane can be visualized by the dry-cleaving technique (Roos *et al.* 1985). We observed that the distribution of surface proteins on hepatoma cells reacting with the lectins concanavalin A and wheat-germ agglutinin, and therefore probably most glycoproteins, coincided with cortical filaments. This observation was recently confirmed for a particular glycoprotein, the epidermal growth factor (EGF) receptor on A431 carcinoma cells (Wiegant *et al.* 1986). On these cell types, and under the

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conditions used, the ligands did not induce a redistribution of the bound surface proteins. We have now applied the same technique to integral plasma membrane H-2 molecules on mouse lymphoma cells. These proteins form patches and caps after binding to anti-H-2 antibodies only if followed by a second layer of anti-Ig antibodies (Taylor *et al.* 1971). Using immunofluorescence techniques, involvement of the cytoskeleton in this redistribution was suggested by core distribution of the cytoskeletal proteins actin (Bourguignon & Singer, 1977; Bourguignon *et al.* 1978*a*), α-actinin (Geiger & Singer, 1979), fodrin (Levine & Willard, 1983) and, in some reports, myosin (Bourguignon & Singer, 1977; Bourguignon *et al.* 1978*b*; Braun *et al.* 1978; Geiger & Singer, 1979).

A detergent-resistant association between cell-surface glycoproteins and the cytoskeleton can be induced by cross-linkage of the glycoproteins, as was first demonstrated by Flanagan & Koch (1978) for cell-surface Ig. Histocompatibility antigens on lymphoid cells are not, or not strongly, bound to the cytoskeleton (Koch & Smith, 1978) and can be dissolved by detergent treatment (Mescher *et al.* 1981). Crosslinking of these antigens by a double layer of antibodies seems to induce linkage with the cytoskeleton, as is indicated by the complete inhibition of their lateral diffusion (Woda & Gilman, 1983; Woda & McFadden, 1983; Edidin & Zuniga, 1984). This linkage must be formed by a reaction with component(s) located either within the plasma membrane or very close to its inner face, since immobilization also takes place when the cytoplasmic domain of H-2 antigens is truncated to only four amino acids (Edidin & Zuniga, 1984). At present, the nature of this linkage is far from clear. Comigrating α-actinin and fodrin were seen to be localized closer to the membrane than actin and myosin, and are therefore likely candidates for linker proteins. The resolution of immunofluorescence, however, is not sufficient to draw any conclusions at this point.

In this paper we show that prior to binding of antibody, H-2 molecules are preferentially located over filaments, indicative of a least a weak interaction, even though no detergent-resistant linkage between H-2 antigens and the cytoskeleton was detected. Incubation with the primary antibody did not induce a detectable change in distribution of H-2 antigens in the plane of the membrane, nor in morphology of the isotropic cortical network. However, detergent-resistant linkage of at least a part of the H-2 antigens was induced. Under capping conditions, H-2 antigens were concentrated into patches and, in some of the cells, into caps. This was accompanied by a local rearrangement of the cortical network from an isotropic distribution to a parallel arrangement of filaments immediately adjacent to the inner face of the plasma membrane and a further increased linkage to the detergent-resistant cytoskeleton.

Materials and methods

Cells

GIL4 mouse leukaemia cells (Graffi virus-induced T-cell leukaemia) were maintained as ascites by serial intraperitoneal (i.p.) transplantation in C57BL/6 mice. Suspensions were prepared and kept at room temperature to prevent possible cold-induced effects on the cytoskeleton.

Antisera

Polyclonal mouse anti-H-2 serum (C3H anti-C3H.B10) was kindly provided by P. Demant and M. Oudshoorn-Snoek (Snoek *et al.* 1979), monoclonal rat anti-H-2 and anti-T200 antibodies were a generous gift from E. Martz (University of Massachusetts, Amherst, USA). As secondary antibodies we used goat anti-mouse IgG (GAM) and rabbit anti-rat IgG (RARa) conjugated to fluorescein isothiocyanate (FITC) or to tetramethylrhodamine isothiocyanate (TRITC) (Nordic Pharmaceutical, Tilburg, The Netherlands), and GAM conjugated to 5 nm gold particles (Janssen Pharmaceutica, Beerse, Belgium). As a third layer, rabbit anti-goat IgG conjugated to FITC (Nordic) was used. For quantification of bound immune complexes we applied ^{125}I-labelled sheep anti-mouse Ig and ^{125}I-labelled sheep anti-rat Ig (Amersham Int. plc, Amersham, UK).

Immunofluorescence

Distribution of H-2 antigens over the complete cell surface was analysed by indirect immunofluorescence. The cells were incubated with mouse or rat anti-H-2 antibodies followed by incubation with GAM/FITC or RARa/FITC. Incubations were performed for 30 min at room temperature unless otherwise stated. In control experiments anti-H-2 antibodies were omitted or replaced by normal mouse serum. The cells were fixed with 3·7% paraformaldehyde (PF) either before or after the primary antibody labelling, or after incubation with the secondary antibody. Antisera were diluted with PBS containing 0·1% bovine serum albumin (BSA). Each incubation was followed by three washes with PBS. The distribution of H-2 antigens as rings, patches or caps was observed and quantified with a Zeiss epifluorescence microscope.

The relation between H-2 antigens and T200 glycoproteins was studied in co-capping experiments. After induced capping of H-2 antigens by 30-min incubations at room temperature with mouse anti-H-2 serum and GAM/FITC, cells were fixed with PF and subsequently treated with rat anti-T200 and RARa/TRITC. For the reciprocal experiment, cells were first incubated with rat anti-T200 and RARa/TRITC, fixed with PF and treated with mouse anti-H-2 and GAM/FITC.

Dry-cleaving and electron microscopy

Dry-cleaving was performed according to Mesland & Spiele (1983). After the final incubation the cells were washed with PBS, fixed with 0·1% glutaraldehyde (GA), rinsed with buffer and allowed to settle on poly-L-lysine-coated Formvar/carbon-covered grids. Then they were treated with 0·1%

tannic acid, postfixed with 0·1 % OsO_4 for 5 min at 4°C, stained with uranyl acetate, dehydrated with ethanol and critical-point dried. Great care was taken to dry the ethanol used for the last dehydration step and the liquid CO_2 used for critical point drying. For dry-cleaving, the grids were positioned cell-side down on the adhesive side of a piece of Scotch tape, gently pressed and removed again, leaving the greater part of each cell on the tape. The grids were immediately viewed in a Philips EM301 electron microscope, or stored for short periods in a dry environment. Stereomicrographs were taken at a tilt angle of ±6°C. Batches of the cells used for dry-cleaving were also routinely embedded and thin sectioned.

Triton X-100 extraction

Extraction with Triton X-100 (1 %) for 30 min at room temperature, was performed before, between or after incubations with antisera. Detergent extraction was always preceded by a mild fixation with PF (2 % for 15 min) in order to preserve the fine structure without cross-linking the antigens to elements of the cytoskeleton. The secondary antiserum was conjugated to FITC for immunofluorescence, 5 nm gold particles for EM on dry-cleaved or thin-sectioned samples, or with ^{125}I for quantification of bound immune complexes in a gamma counter.

Results

Fluorescence microscopy and EM on thin sections

A redistribution of H-2 antigens was induced when unfixed cells were incubated at room temperature with, consecutively, anti-H-2 and GAM antisera. In about 25 % of the cells the immune complexes were concentrated into caps, while on the remaining cells large patches were induced (Fig. 1A–D). A similar redistribution occurred when 5 nm gold particles were conjugated to the secondary antiserum, as was demonstrated with immunofluorescence using RAG/FITC as tertiary antibody. Cap formation was slow and detectable only after 10–15 min incubation with the secondary antiserum, while small patches were already formed within 5 min. By EM on thin sections we observed that on capped cells practically all gold-labelled immune complexes were concentrated at the caps, whereas other parts of the cell surface were devoid of gold particles (Fig. 1B). This was accompanied by a change in distribution of coated pits and vesicles. Originally distributed all over the cell surface, they also were now concentrated at the caps, where gold-labelled immune complexes were endocytosed (Fig. 1A). A thick layer of filamentous material was often present under the caps (Fig. 1B). Under patches the thickness of the filamentous layer varied, as elsewhere, from 50–150 nm (Fig. 1D). After fixation before or between incubations, fluorescent immune complexes were seen as smooth or finely punctate rings (Fig. 1E,G), while in thin sections gold particles were distributed singly or in very small groups (Fig. 1F,H). On thin sections a difference was observed between cells fixed with GA or with PF (before or between the immune incubations). In the case of GA fixation, groups of more than five particles were never seen (Fig. 1F), while after PF fixation groups of up to 10–15 particles were also present (Fig. 1H). In all control experiments we observed no fluorescence and virtually no gold particles.

Dry-cleaved preparations

Dry-cleaving of immunogold-labelled cells yielded preparations of plasma membrane fragments, generally sheets of 2–3 μm^2. Gold particles remained attached to the outer face of these membrane fragments, while the cytoplasmic side was occupied by elements of the cortical network. Since the cells adhered in a random fashion to the grid (Mesland & Spiele, 1983), we expected to obtain fragments of random parts of the cell surface, including caps. Cleavage of caps, however, was hampered by their weak adherance to the grids, due to their irregular villous surface. Thus the number of cleaved caps could not be related to the number of capped cells. The few cleaved caps could be recognized as such by a concentration of gold particles and the presence of many endocytotic vesicles. Areas away from caps, on the contrary, were practically devoid of gold label and coated pits. On cleaved fragments of non-capped cells patches of gold particles were practically always observed.

Morphology of cortical network

In stereomicrographs of dry-cleaved labelled cells three levels of depth were discerned (Fig. 2): one formed by the gold particles at the outer face of the plasma membrane; one at the inner face of the plasma membrane, locally recognizable by flat or somewhat convex areas decorated with regular clathrin networks; and, at the cytoplasmic side, a layer of cortical material limited by the plane of cleavage. Since the plane of cleavage was not regular, the thickness of the latter layer varied. Thicker areas contained filaments together with concentrations of granular electron-dense structures, probably ribosomes and associated proteins, and, locally, smooth and coated vesicles; at thinner areas only the filamentous network immediately adjacent to the plasma membrane remained present. This network was mainly formed by interconnected filament strands of approx. 5–7 nm diameter and 50–70 nm in length, with short side branches often pointing towards the plane of the membrane. Intermediate filaments and microtubules were only very rarely observed. At the plane of the membrane, the electron density was not uniform: areas of somewhat increased density were seen to be congruent with the underlying filamentous network. These darker domains were also present in equal amount and distribution in control cells, and thus do not represent immune complexes attached to the outer

surface of the cells. The short side branches of filaments generally pointed towards these opaque domains.

The cortical network of cells fixed before or immediately after incubation with the first antiserum was isotropic without any prevailing direction of the filaments (Figs 2, 3A). No difference in the pattern of the filamentous network was observed when cells fixed with GA or PF were compared. However, when unfixed cells were incubated with both primary and secondary antibodies, a rearrangement of filaments was observed in some of the cleaved fragments (Figs 3B, 4). Long filaments were seen to run parallel to each other at a very short distance from the plane of the plasma membrane. Together they covered either narrow strips when arranged into bundles (Fig. 3B), or a broad band of the cytoplasmic face of the membrane (Fig. 4). In both forms the rearranged filaments formed a very thin layer, generally not more than one filament thick, as could be seen in thicker areas where they were covered by isotropic filaments and granular material. The parallel filaments (diameter approx. 6–7 nm) were

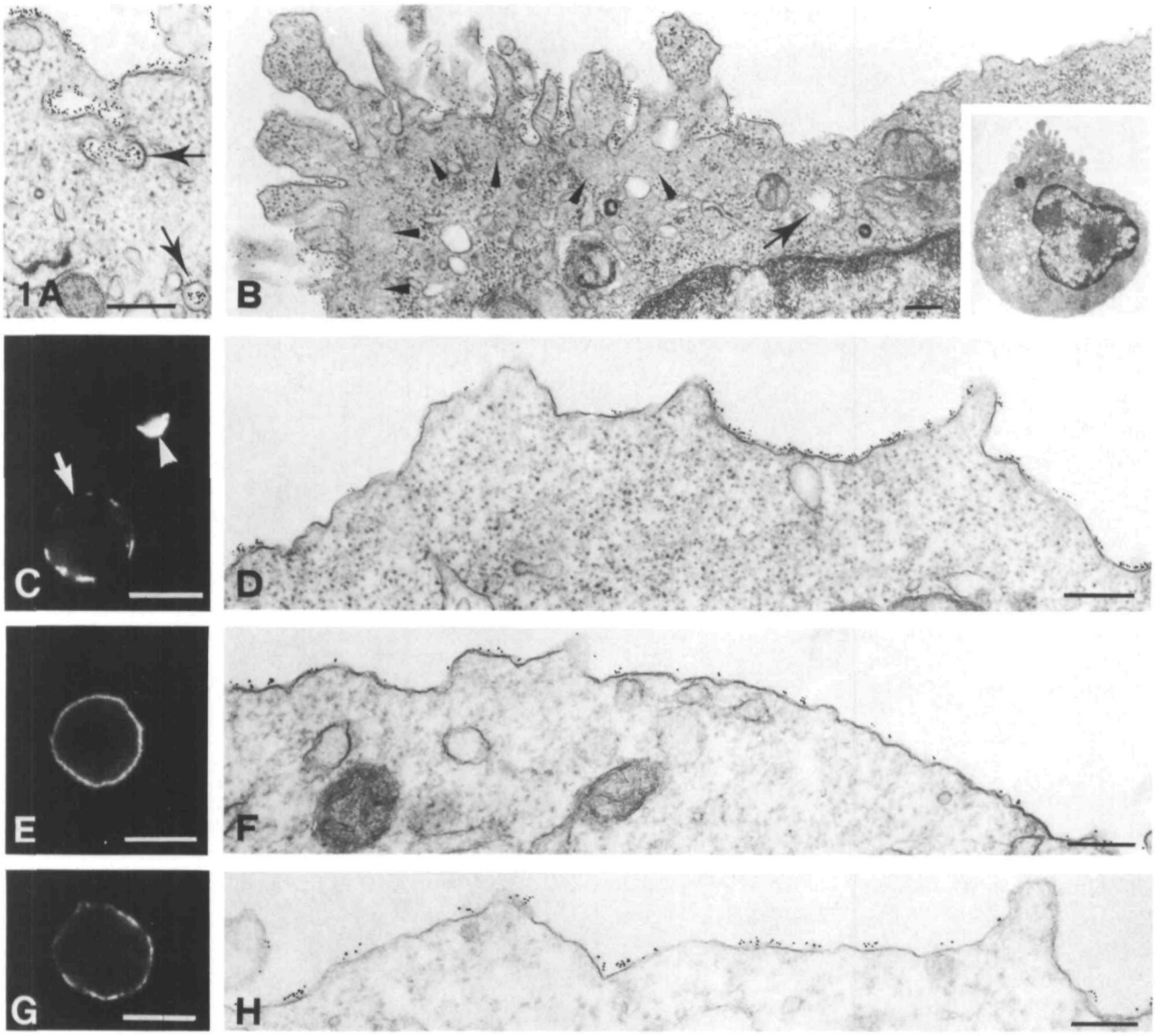


Fig. 1. Distribution of H-2 antigens on GIL4 mouse leukaemia cells. A–D. Fixation after incubations with anti-H-2 serum and GAM.G5 (A,B,D) or GAM/FITC (C). Immune complexes are redistributed into patches (C (arrow) and D) or into caps (A–C (arrowhead)), where they are endocytosed (A,B, arrows). Gold label is practically exclusively concentrated at the cap (B and inset). Filamentous material is concentrated under the cap (B, arrowheads). E. After prefixation with PF, the immune complexes form a smooth ring. F–H. Fixation after incubation with anti-H-2 serum but before incubation with GAM. Gold particles are distributed singly or in small groups when cells are fixed with GA (F). Punctate fluorescent rings (G) and more distinct groups of gold particles (H) are seen when PF is used for fixation. Bars: C,E,G, 10 μm; A,B,D,F,H, 0·2 μm. A,D–H, ×50 000; B, ×27 000; inset, ×3800.

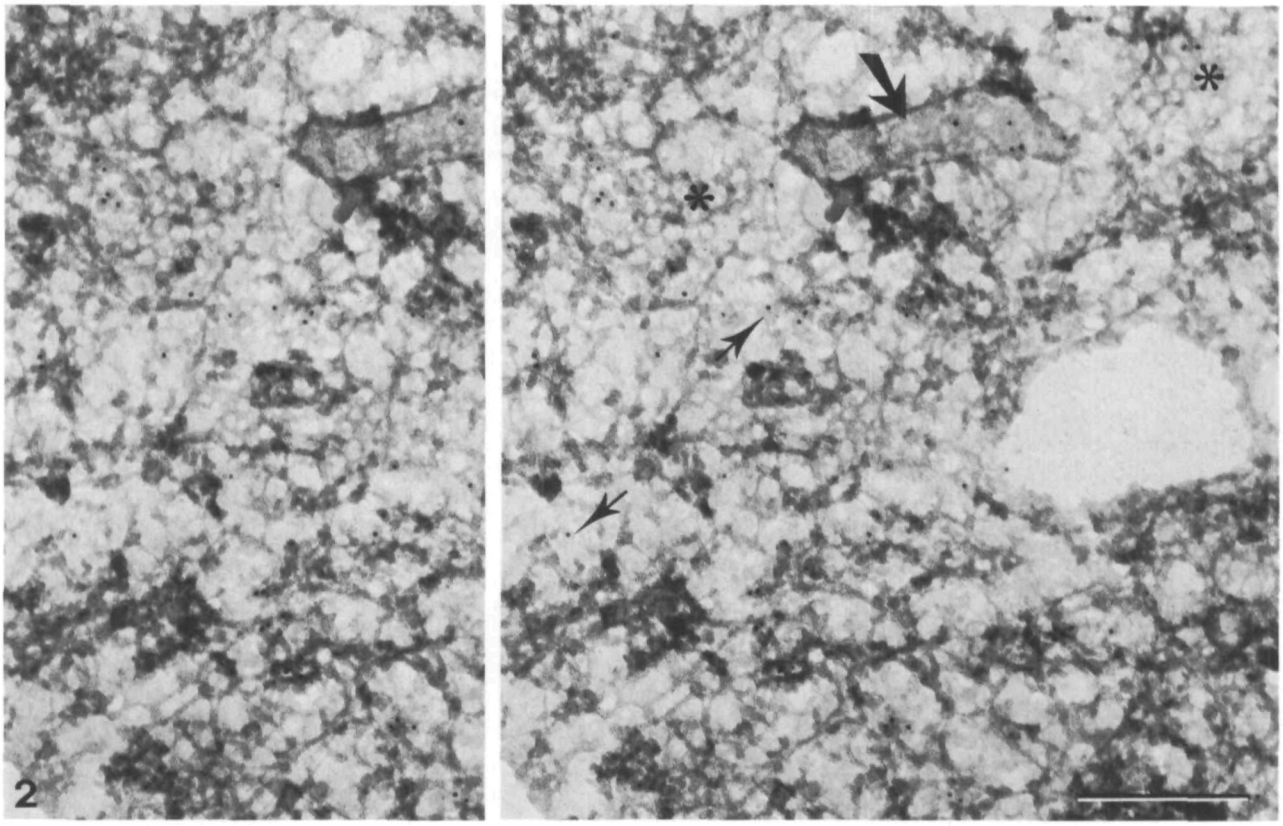


Fig. 2. Dry-cleaved cell fixed with GA before incubations with anti-H-2 serum and GAM.G5. The left part is reproduced in stereo, viewing direction from inside the cell. Gold particles are attached to the outer face of the plasma membrane. The inner face is locally decorated by regular clathrin networks (asterisks) and is covered by an isotropic filamentous network to which ribosomes and a smooth vesicle (large arrow) are attached. Gold particles are localized to areas of somewhat higher electron density at the plane of the membrane (small arrows) over or adjacent to elements of the filamentous network. Bar, 0·2 μm; ×112 500.

interconnected by cross-bridges and had short side branches. Areas immediately adjacent to parallel filaments were covered with an isotropic network, into which the bundles could be seen to fan out. In control cells a parallel arrangement of filaments was never observed.

Distribution of H-2 antigens

The distribution of gold particles over larger areas of the membrane, as seen in dry-cleaved preparations, corresponded to that seen in thin sections. Thus after GA fixation, either before or after incubation with the first antiserum, particles were dispersed over the cell surface both as single particles and in small groups of up to 10 particles (Figs 2, 5A). When PF was used, groups of up to 25–30 particles were present next to freely dispersed particles (Fig. 5B). These groups, however, were much smaller than patches induced by incubation with both antisera before fixation (Figs 5C, 7). Under the latter conditions free particles were rare. Since only part of the surface of each cleaved cell remained attached to the grid, the density and distribution of gold particles varied widely. Generally, one or more patches were present, while on other cleaved sheets gold particles were virtually absent. Large sheets devoid of both gold particles and coated pits or vesicles most probably represented parts of the plasma membrane away from the caps (Fig. 4). On the other hand, areas with large concentrations of gold particles together with coated pits, and both coated and smooth vesicles filled with gold particles, were probably derived from caps (Fig. 6).

Relation between labelled H-2 antigens and cortical structures

Irrespective of the mode (PF or GA) and moment of fixation (before, between or after immune incubations), the gold label was always localized to areas in the plane of the membrane of somewhat higher electron density situated immediately over or adjacent to elements of the filamentous network (Figs 2, 6, 7). Gold particles were rarely localized over coated areas when cells were fixed before or between incubations with the antisera.

When concentrated into large patches the arrangement of the gold particles matched that of the filamentous network adjacent to the plane of the membrane. At the plane of the membrane, concentrations of electron-

dense material were seen to underly patches. Patches were present both over isotropic parts of the network and over bundles of parallel filaments (Fig. 7). At areas presumably derived from caps the network was generally isotropic (Fig. 6). Relatively large areas with parallel filaments were usually devoid of gold particles and coated pits, and most probably represented areas away from caps. The thickness or density of the cortical network under patches or caps varied as elsewhere. This does not exclude concentrations of cytoskeletal material in these areas, since these may have been detached during cleavage.

Linkage of H-2 antigens to the cortical network

The formation of detergent-resistant linkages between H-2 molecules and the cytoskeleton was investigated by extraction of cells with Triton X-100 before, between or after incubations with antisera. Secondary antisera

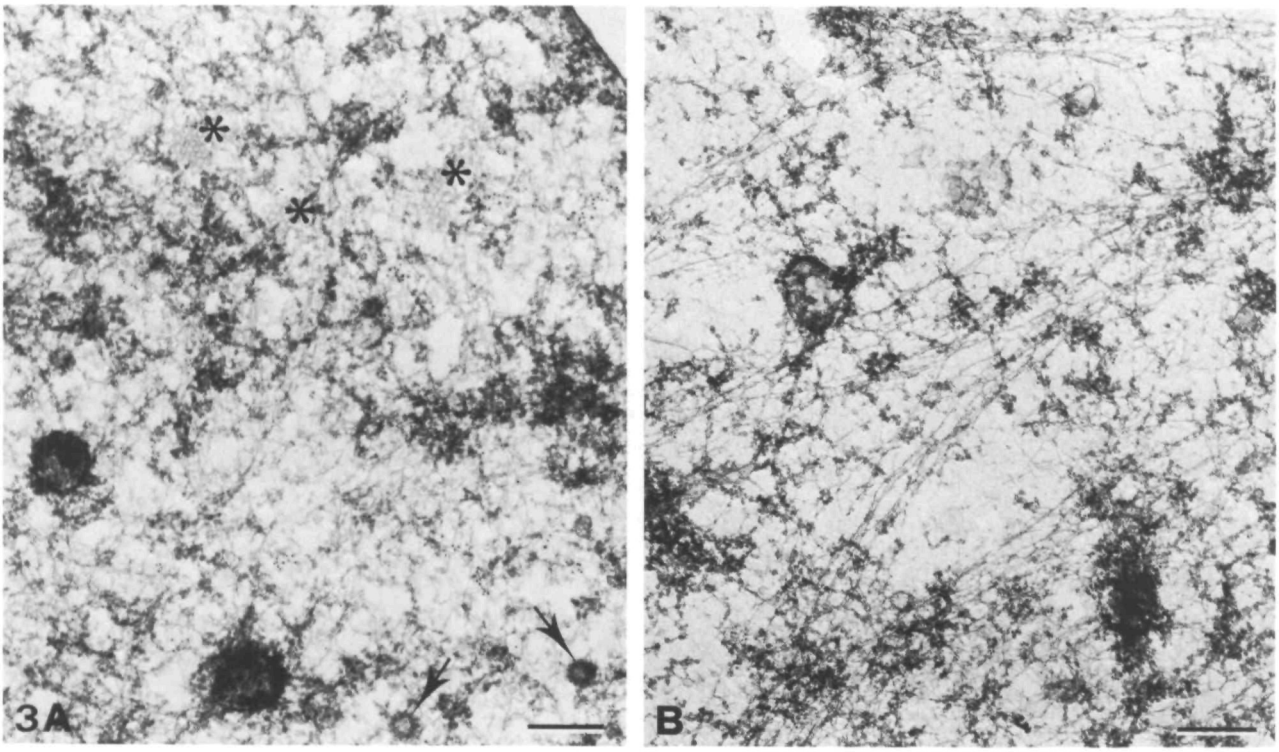


Fig. 3. Dry-cleaved preparations of cells fixed with GA before (A) or after (B) incubations with anti-H-2 serum and GAM.G5. A. After prefixation, the filamentous cortical network is isotropic; coated areas (asterisks) and coated pits (arrows) are regularly distributed. B. A rearrangement of the network into bundles of parallel filaments is induced by immune incubations of unfixed cells. In this area no coated pits are present. Bars, 0·2 μm; ×50 000.

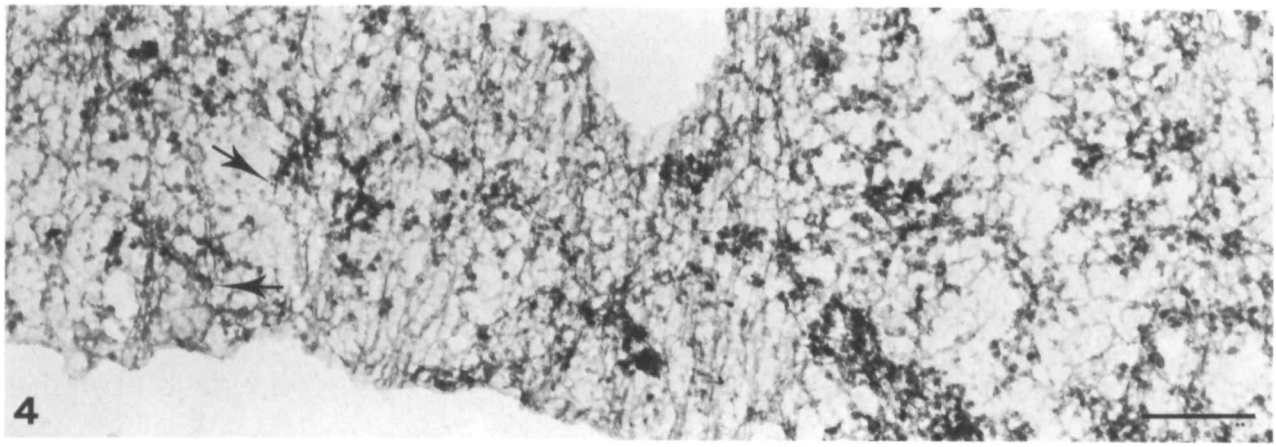


Fig. 4. Incubation of unfixed cells with anti-H-2 serum and GAM.G5. A broad band of parallel filaments is formed next to an area covered by an isotropic network. Few gold particles (arrows) and no coated areas are present. Bar, 0·2 μm; ×75 000.

were labelled with gold particles, FITC or ^{125}I. In order to preserve sufficiently the fine structure of the filamentous network, Triton extraction was preceded by a mild fixation with PF.

When detergent extraction preceded incubations with both antisera, only a few gold particles were observed on thin sections and dry-cleaved preparations (Fig. 8E,I). When the cells were extracted after incubation with anti-H-2 serum, but prior to exposure to the secondary antibody, more particles were attached to fibrillar structures (Fig. 8F,J). Patches and caps formed by incubation with both antisera were resistant to subsequent detergent extraction (Fig. 8G,H,K). In all instances the plasma membrane and intracellular membranes could not be detected, while the overall structure of cell outline, cortical network, cytoplasmic structures and nucleus remained intact. On control cells, not incubated with the primary antiserum, virtually no gold particles were observed.

For EM the polyclonal mouse anti-H-2 serum was used. Since linkage could depend on the slight clustering of the immune complexes observed when cells were

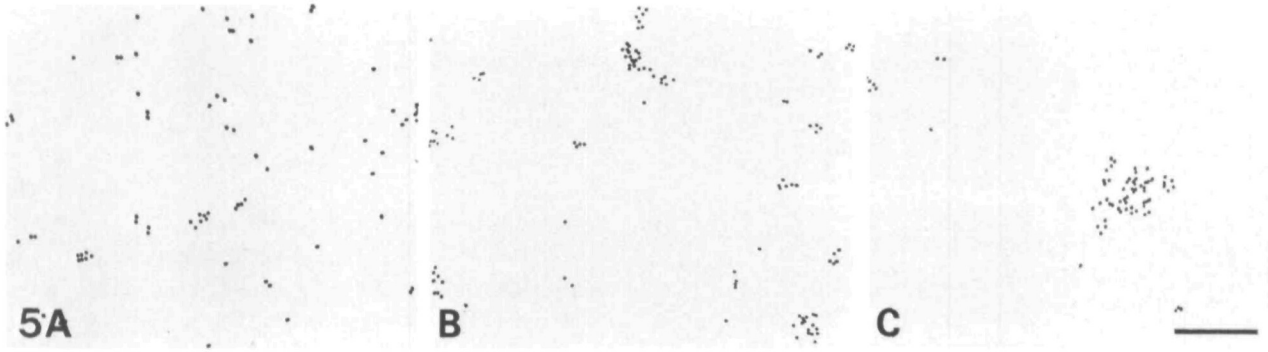


Fig. 5. Distribution of gold particles on dry-cleaved cells. Gold particles are marked on transparent sheets overlying ×112 500 enlarged micrographs. Reproduced areas are fully covered by intact plasma membrane sheets. A. Prefixation with GA; B, prefixation with PF; C, fixation after immune incubations. After prefixation with PF (B), the gold particles are more clustered than after prefixation with GA (A). On unfixed cells, immune complexes are concentrated into distinct patches (C). Bar, 0·2 μm; ×50 000.

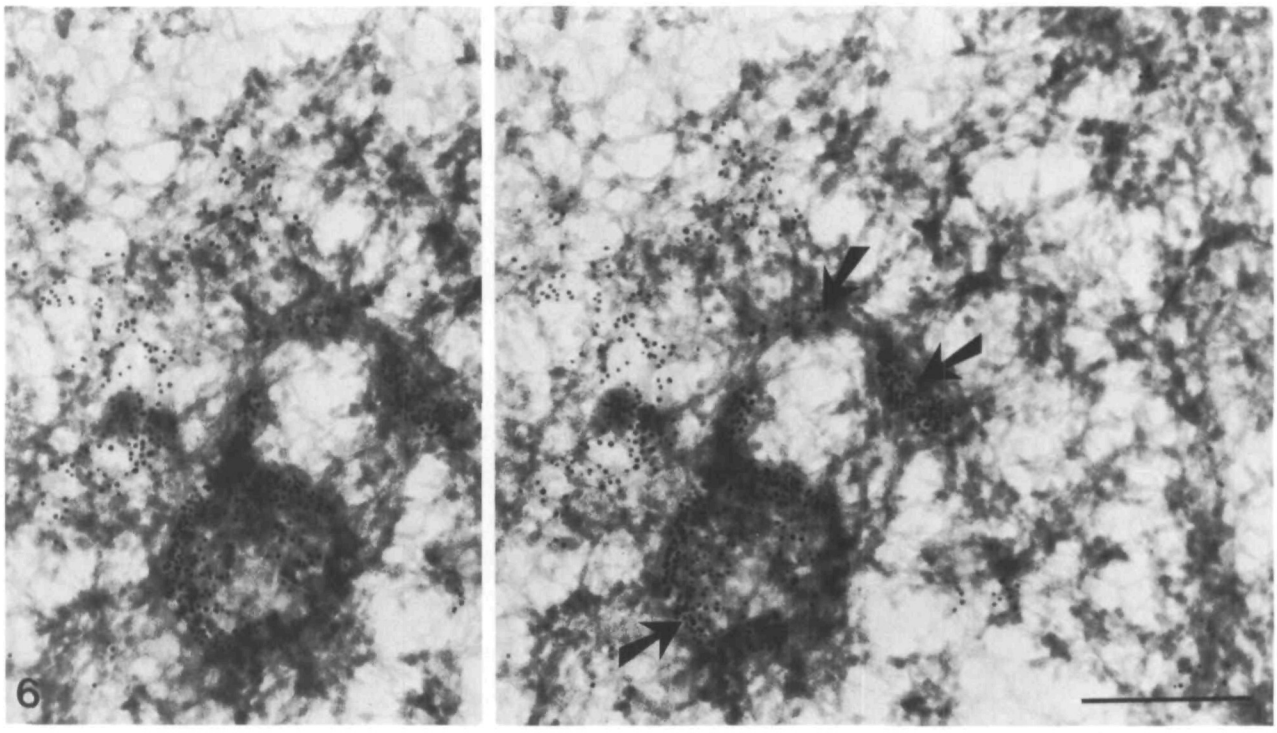


Fig. 6. Dry-cleaved preparation of part of a cell incubated with both antisera before fixation. The left part of the micrograph is reproduced in stereo. A large concentration of gold particles is attached to the outer face of the plasma membrane. At the cytoplasmic side, smooth round and tubular vesicles containing endocytosed gold particles (arrows) are embedded in the isotropic network. This area most probably represents part of a cleaved cap. Bar, 0·2 μm; ×112 500.

fixed with PF before or after incubation with this antiserum, we also used monoclonal rat anti-H-2 antibodies for fluorescence microscopy and radioactive quantification. Fluorescent immune complexes were not observed when detergent extraction preceded the immune incubations (Fig. 8A), while positive fluorescence was seen when detergent treatment was performed between or after the immune incubations (Fig. 8B–D). The fluorescent rings were finely punctate when Triton treatment was performed after incubation with the polyclonal anti-H-2 serum (Fig. 8B), especially when compared with the smooth rings formed by incubation with monoclonal anti-H-2 antibodies prior to Triton extraction (Fig. 8C). Fluorescent caps and patches clearly resisted Triton extraction (Fig. 8D).

Reactions with ^{125}I-labelled secondary antibody (Table 1) confirmed that Triton X-100 extraction prior to immune incubations solubilized practically all H-2 antigens. Detergent resistance of about 40–50 % of the H-2 antigens was induced by reaction with anti-H-2 antibodies (both mono- and polyclonal). Thus even after the minimally cross-linking reaction with monoclonal antibodies, and without detectable movement of the H-2 antigens, a detergent-resistant linkage of about 40–50 % of the H-2 antigens to filamentous structures was induced. Reaction with a secondary antibody further increased detergent resistance to values between 60 % and 80 %.

Relation between H-2 antigens and T200 glycoproteins

The putative role of the T200 glycoprotein as a linker between capping cell surface molecules and the cytoskeleton (Bourguignon *et al.* 1985) was investigated by cocapping experiments. We did not observe cocapping of H-2 antigens after induced capping of T200 glycoproteins, nor of T200 when H-2 molecules were concentrated into caps (Fig. 9). In both cases the last detected antigens formed smooth fluorescent rings over the complete surface of capped cells. Cocapping of a part of the molecules could not be excluded, since at the capped pole their fluorescence was often somewhat stronger than elsewhere over the cell body. However, this could also be due to the increase in net amount of plasma membrane at the capped pole, where the surface generally is very villous.

Discussion

Dry-cleaving of cells yields preparations consisting of the cortical part of the cytoskeleton, the overlying plasma membrane and associated structures. The involvement of the cortical part of the cytoskeleton in capping of cell surface molecules, demonstrated by comigration of certain cytoskeletal elements, is reflected in thin sections by concentrations of filamentous material under caps and sometimes under patches. These concentrations are directly adjacent to the inner face of the plasma membrane, but can also extend deeper into the cytoplasm. Since in dry-cleaved preparations the thickness of the cortical layer was determined by Mesland & Spiele (1983) to be about 50 nm, this technique will give information exclusively about the outermost part of the skeleton.

Several models have been proposed for the interaction between cytoskeleton and plasma membrane during capping. In all models a specific organization of filaments associated with the inner face of the plasma membrane is necessary for directed movement of cell

Table 1. *Detergent resistance of H-2 antigens*

Incubations			Pellet* (cts min^{-1})	% cts min^{-1} in TX-treated pellet
PF.TX	– MAH-2†	– 125I.ShAM	13 293	14·6
PF.–	– MAH-2	– 125I.ShAM	91 353	
MAH-2	– PF.TX	125I.ShAM	54 143	50·1
MAH-2	– PF.–	– 125I.ShAM	108 149	
MAH-2	– 125I.ShAM	– PF.TX	54 389	79·4
MAH-2	– 125I.ShAM	– PF.–	68 466	
PF.TX	– RaAH-2‡	– 125I.ShARa	3 955	3·8
PF.–	– RaAH-2	– 125I.ShARa	104 954	
RaAH-2	– PF.TX	– 125I.ShARa	57 545	44·9
RaAH-2	– PF.–	– 125I.ShARa	129 663	
RaAH-2	– 125I.ShARa	– PF.TX	48 508	62·1
RaAH-2	– 125I.ShARa	– PF.–	78 076	

Cells were fixed (PF), detergent-treated (TX) and incubated with antibodies at room temperature as described in Materials and methods. After the final washing, the pellets were suspended in PBS and the radioactivity was counted.

* Values are the mean of duplicates, corrected for non-specific binding of ^{125}I-labelled secondary antiserum to cells not incubated with the first antiserum under the same conditions.

† Polyclonal mouse anti-H-2 antibodies.

‡ Monoclonal rat anti-H-2 antibodies.

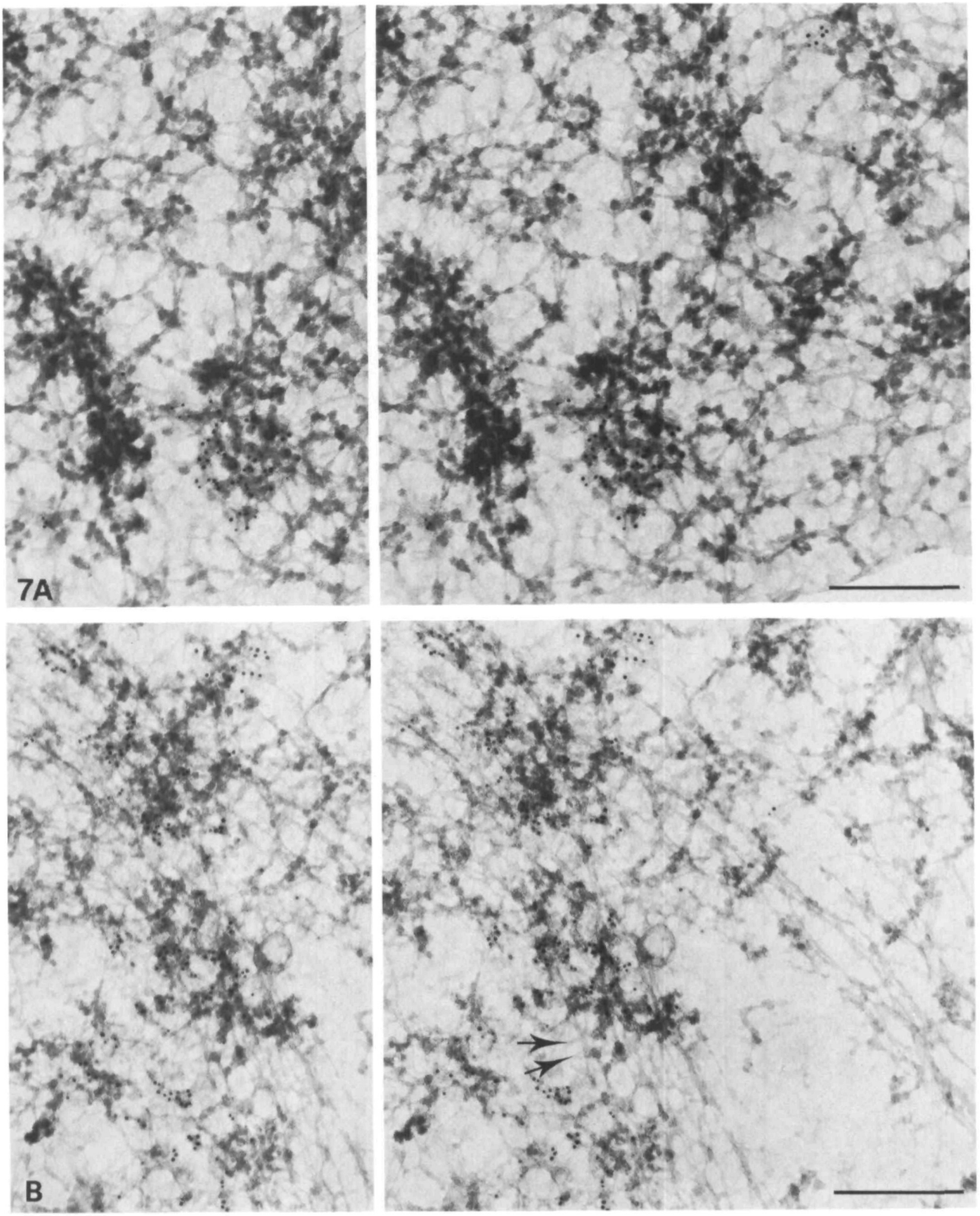
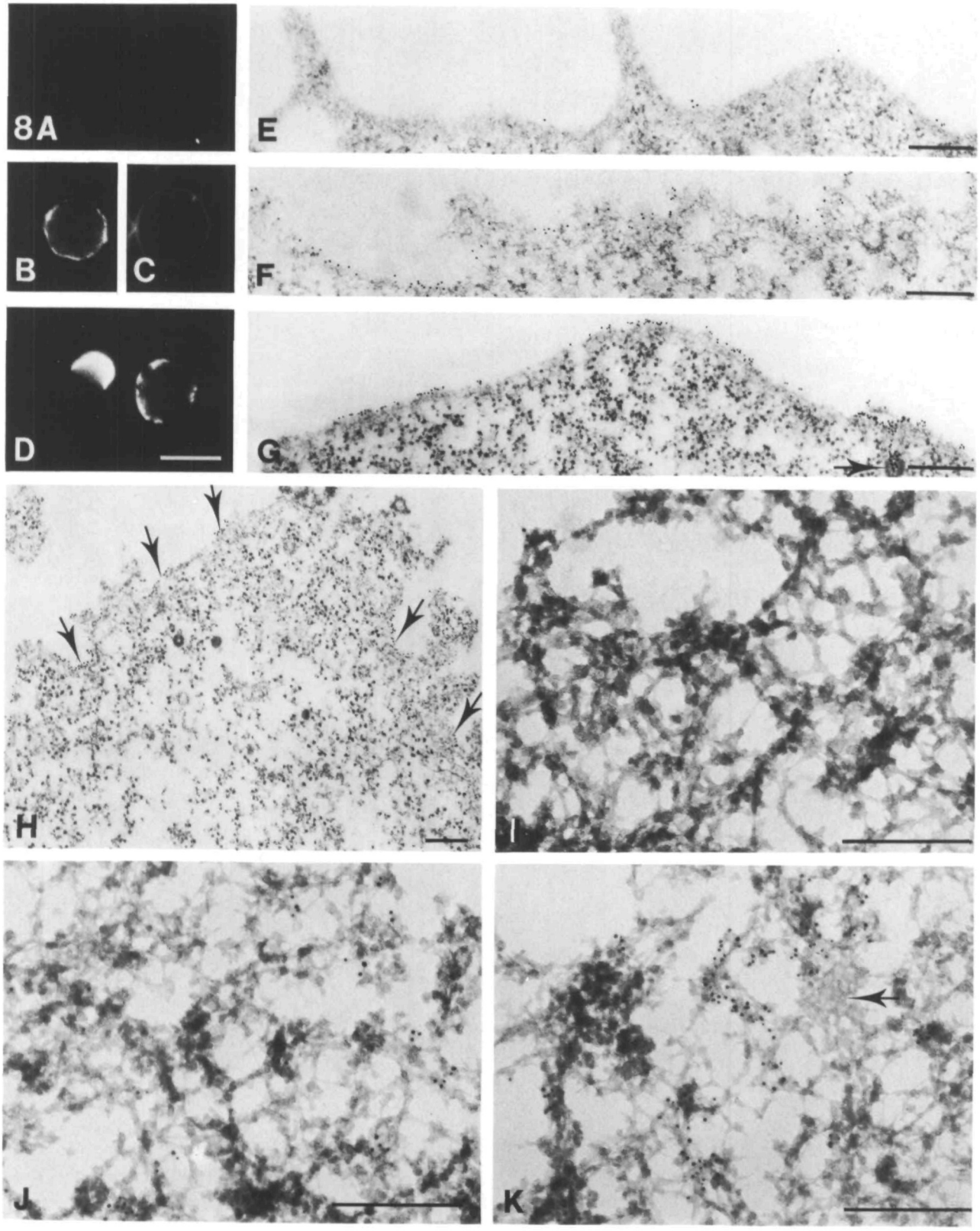


Fig. 7. Dry-cleaved preparations of cells incubated with anti-H-2 serum and GAM.G5 before fixation. The left parts of the micrographs are reproduced in stereo. Gold particles concentrated into patches overlie an isotropic filamentous network (A) and bundles of parallel filaments (B). The parallel filaments form a thin layer at a very short distance from the inner face of the plasma membrane. They are interconnected and have short side branches that point towards the plasma membrane (arrows). Electron-dense material is concentrated at the plane of the membrane under the patches. Bars, 0·2 μm; ×112 500.

surface molecules. Some models suggest that a direct or indirect linkage between surface molecules and the cytoskeleton is essential for capping (Bourguignon & Bourguignon, 1984). In that case, it can be expected that filaments of the cortical network responsible for displacement of patches will be organized as bundles that at least in part colocalize with patched surface molecules. Other models propose that capping is brought about by changes in the shape of the cell, such as waves on the cell surface (Oliver & Berlin, 1982) or a



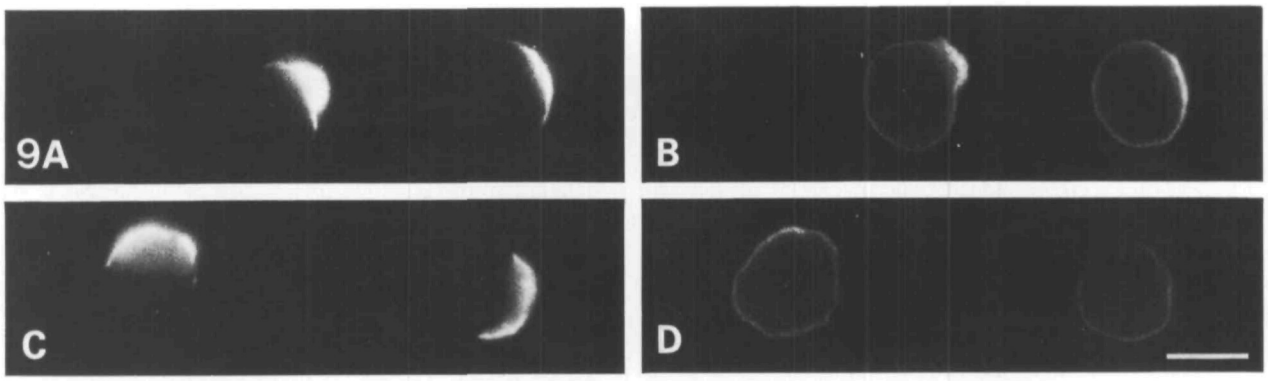


Fig. 9. Relation between H-2 antigens and T200. A,B. On cells with induced H-2 caps (A), T200 remains distributed over the complete cell surface (B). C,D. After induction of caps of T200 (C), H-2 antigens remain distributed over the complete cell surface (D). The cells are fixed after induction of caps by the first antiserum. Note that at the capped pole the fluorescence of antigens detected after fixation can be more brilliant than elsewhere (B). Bar, 10 μm.

circular constriction (Loor, 1981). These will be induced by rearrangement and contraction of membrane-associated filaments mainly localized next to and not under patches or caps.

The cortical skeleton of mouse lymphoma cells consists of an isotropic network of filaments, as already described by Mesland & Spiele (1983) for lymphoid cells. When capping of cell-surface H-2 molecules was induced, a local rearrangement from the isotropic network into parallel filaments immediately adjacent to the inner face of the plasma membrane was observed. This rearrangement is probably similar to that observed by Michaels *et al.* (1984) on replicas of the inner surface of sonicated capped cells. H-2 molecules concentrated into patches were overlying and attached to both isotropic and rearranged parts of the network. Since EM observations reflect the situation at one moment, it is not certain whether those patches that overlie parallel filaments are in the process of being moved towards the cap. However, since the majority of the patches overlie isotropic parts of the network, a rearrangement does not seem to be required for the formation or consolidation of patches, but may be necessary for their displacement toward the capping area. Large areas with parallel filaments were generally devoid of H-2 molecules and of coated pits. Since in thin sections it was seen that in capped cells practically all H-2 antigens and coated pits were present at the capped pole and not elsewhere at the cell surface, these large sheets of plasma membrane overlying parallel filaments probably represented parts of the cell surface next to or away from caps. It seems likely that these filaments form part of a structure similar to a contraction ring that is either pushing the patches towards the capping pole, or necessary to keep the immune complexes concentrated at the cap.

Under all conditions, the vast majority of H-2 molecules were localized in conjunction with elements of the filamentous network. Incubation with the first antibody did not induce a detectable displacement of H-2 molecules with respect to each other or to elements of the filamentous network. Moreover, the structure of the isotropic network did not change either. Nevertheless, incubation with the first antibody induced resistance to extraction with Triton X-100 of about 50 % of the H-2 molecules that originally were practically all solubilized by Triton treatment. This indicates that for formation of a linkage between Triton X-100-resistant skeletal elements and H-2 molecules rearrangement of either H-2 molecules or elements of the cytoskeletal network is not necessary. Formation of a linkage between H-2 antigens and the cytoskeleton after reaction with monoclonal antibodies against H-2 antigens was also described by Henis & Gutman (1983). They found with immunofluorescence that this immobilization was always accompanied by the formation of clusters of less than 1 μm of the immune complexes. However, in our experiments linkage of H-2 antigens to the cytoskeleton by reaction with monoclonal antibodies was not accompanied by formation of these

Fig. 8. Effect of Triton X-100 extraction on detection of H-2 antigens; fluorescence microscopy (A–D), thin sections (E–H) and dry-cleaved preparations (I–K). A,E, I. Extraction before incubations with anti-H-2 serum. No fluorescent cells (A) and only very few gold particles (E and I) are present. B,C,F,J. Extraction after incubation with polyclonal (B,F,J) on monoclonal (C) anti-H-2 antibodies. After reaction with polyclonal antibodies, fluorescent rings are punctate (B), those formed by reaction with monoclonal antibodies are smooth (C). Dispersed gold particles are attached to filamentous structures (F,J). D,G,H,K. Extraction after incubations with anti-H-2 serum and GAM. Immune complexes concentrated into caps (D,H arrows) or patches (D,G,K) remain attached to filamentous structures. Plasma membrane and intracellular membranes have disappeared; cortical filaments, ribosomes and clathrin networks (G,K arrows) remain intact. Bars: A–D, 10 μm; E–K, 0·2 μm. E–G, ×57 000; H, ×35 000; I–K, ×112 500.

patches. The detergent-resistant fluorescent rings remained completely smooth, even after mild fixation with formaldehyde, which does not fully immobilize immune complexes.

The present observations cannot answer the question of whether the linkage between complexed H-2 molecules and the cytoskeleton is direct or indirect *via* molecules embedded in or associated with the plasma membrane. Bourguignon *et al.* (1985) proposed that the connection between patched receptors and the cytoskeleton might be formed by a complex of the T200/gp 180 glycoprotein and fodrin, which in its turn can bind to actin (Glenney *et al.* 1982). If this should be the case, cocapping of T200 molecules and H-2 antigens is to be expected. However, contrary to their findings (Bourguignon *et al.* 1984), we did not observe cocapping of H-2 antigens with capped T200 glycoproteins, nor of T200 with capped H-2 antigens. Cocapping of a minor portion of the molecules could not be excluded, by using immunofluorescence methods. Characterization of possibly comigrating membrane-associated molecules and of molecules that form the linkage with the cytoskeleton will be carried out with immuno-EM using antibodies against components associated with the inner face of the plasma membrane.

In conclusion we can state that H-2 molecules are always localized in conjunction with elements of the cortical filamentous network; that formation of a linkage with this network can be induced by reaction with anti-H-2 antibodies without any major redistribution of H-2 antigens or of skeletal elements; and, finally, that under capping conditions the filaments immediately adjacent to the plasma membrane are rearranged.

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