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OBSERVATIONS ON TRANSMEMBRANE STRUCTURES OF SURFACE IMMUNOGLOBULIN IN THE PLASMA MEMBRANE OF B LYMPHOCYTES

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We have investigated the possible role of intramembraneous particles as revealed by freeze-fracture electron microscopy in the plasma membrane of B lymphocytes from rabbits and mice as reflections of transmembrane structures of surface immunoglobulin receptor molecules. This was achieved by aggregation of the surface receptors using fluorochrome-conjugated antibodies, fixation and freezing of the cells in 35% glycerol. This procedure resulted in replicas of lymphocytes with well-preserved morphology (no ice-crystals), enabling the study of both protoplasmic and external fracture face in combination with surface receptor markers. It appeared that very small intramembraneous particles (3–6 nm diameter) were selectively clustered under patches of surface receptor label. This phenomenon was found on the external fracture face exclusively and not on the protoplasmic fracture face. 'Classical' intramembraneous particles (6–12 nm diameter) were not involved. We suggest that these small, clustered particles should be interpreted as transmembrane structures of surface immunoglobulin molecules.

Introduction

Endogenous surface immunoglobulin of B lymphocytes is the basic antigen receptor on the surface of these cells (for review, see Warner [1] and Goodman [2]). Surface immunoglobulin is an integral and most probably transmembrane protein as deduced from several considerations: first, it can be solubilized from B lymphocytes by detergents only and readily precipitates if the detergent is removed [3,4]; second, a connection exists between sIg on the surface of the cell and cytoplasmic microfilaments reaching the inner surface of the plasma membrane [5–11]; third, from studies on mRNA encoding for secretory and membrane-bound IgM it appeared that sIgM possesses an 'extra' peptide with a hydrophobic amino acid sequence long enough to span the lipid bilayer of the plasma membrane [12]. Recently, this has also been shown for sIg molecules of the IgA [13] and IgD isotype [14].

In the present study we have tried to demonstrate if surface immunoglobulin was correlated with any intramembraneous structure as visualized by freeze-fracturing, which would indicate the presence of a peptide chain spanning the membrane. The classical approach for this demonstration is by labelling cells with ferritin-conjugated antibodies, freeze-fracturing in low salt media and deep-etching [15]. However, such an approach has some severe drawbacks. First, the method implies the freezing of cells in low-salt media without any cryoprotectant, thus (when using conventional freezing procedures) resulting in severe structural damage to the cells. This means that only minor parts of fracture faces and etched surfaces can be observed. Second, this method shows etched cell surfaces in combination with protoplasmic frac-

ture faces exclusively. It is not possible to display exoplasmic fracture faces in combination with molecules labelling the outer cell surface [16,17].

For these reasons we have applied a new method for the detection of antibodies in replicas [16,17]. This method will be described in detail elsewhere (unpublished data). It enables us to study both protoplasmic and exoplasmic fracture faces in combination with label material on the cell surface without (by the presence of cryoprotectants) structural damage. Using this method we can demonstrate aggregation of intramembraneous particles on the exoplasmic fracture face closely following the pattern of label for surface immunoglobulin.

Materials and Methods

Cells. Spleen lymphocytes from chinchilla rabbits and Swiss random mice were obtained by centrifugation on Ficoll-metrizoate as described previously [18]. The cells thus obtained consisted of at least 95% viable cells, containing about 90% lymphocytes [19]. Lymphocyte cell population consisted of $54 \pm 2\%$ sIg-bearing B lymphocytes for rabbits [18] and $70 \pm 1\%$ for mice (unpublished data).

Antibodies. A polyspecific fluorescein- or rhodamine-conjugated horse anti rabbit immunoglobulin antiserum, polyspecific fluorescein-conjugated rabbit anti mouse immunoglobulin antiserum and rhodamine-conjugated rabbit anti horse IgG were used as described previously [18].

Experimental protocol. All basic incubation conditions were used as optimalized according to our earlier reports [18,19]. In short, 10^7 spleen lymphocytes were equilibrated for 30 min at 37°C in our medium (Hanks' balanced salt solution) in polystyrene tubes. Prewarmed fluorescein-conjugated horse anti rabbit Ig antiserum (final dilution 1:16) was added to rabbit cells and the incubation was allowed to proceed for 5 min. Tubes were then transferred to ice and the cells were washed twice in medium and finally suspended in medium containing rhodamine-conjugated anti horse IgG antiserum (final dilution 1:20). To prevent cold-induced clustering of intramembraneous particles (unpublished results), the cells were warmed up to 37°C for 5 min prior to fixation in 2% glutaraldehyde in Sörensen's buffer as previously described [19]. For mouse cells, fluorescein-conjugated rabbit anti mouse antiserum (1:16) and rhodamine-conjugated anti rabbit Ig antiserum (1:20), respectively, were used.

Freeze-fracturing. For freeze-fracturing glutaraldehyde-fixed cells were washed in medium and suspended in 35% glycerol in distilled water. Freeze-fracturing was carried out in a Balzers' BAE 301 Freeze Etch Unit (Balzers AG, Liechtenstein) at -100°C and a vacuum of 10^{-7} mbar. This apparatus has been supplied with a liquid nitrogen-cooled shield covering the specimen, with holes for electron gun evaporation of Pt/C and C. This shield effectively protects the specimen from contamination, allowing exposure of fractured specimens for 5 min down to -130°C without visible contamination.

Fracturing of specimens frozen in liquid propane cooled by liquid nitrogen was performed according to the double replica technique of Mühlethaler et al. [20], using Polaron gold finder grids and Balzers double replica gold specimen supports. Two nanometers of Pt/C and 15 nm of carbon were evaporated under 45 and 90° angle, respectively, using Balzers electron gun evaporators, controlled by a quartz thin-layer thickness monitor. Grids with replicas were cleaned overnight in commercial bleach, followed by 30% chromic acid for 2 h at 80°C. Replicas were observed and pictures were taken in a Philips EM 300 electron microscope, operated at 80 kV.

Results

The labelling procedure applied here employs the incubation of cells with two subsequent layers of fluorochrome-conjugated antibodies and fracturing of cells frozen in 35% glycerol. Under appropriate conditions no additional marker molecules (e.g. ferritin) are needed since this sandwich of antibodies will provide sufficient mass for detection in electron microscopy. It can be shown as discrete patches of fuzzy material in thin sections of tannic acid/glutaraldehyde-fixed cells (Fig. 1a; compare Roholl et al. [21]).

In freeze-fracture electron microscopy biological membranes are split through their hydrophobic interior. This process generates two different frac-

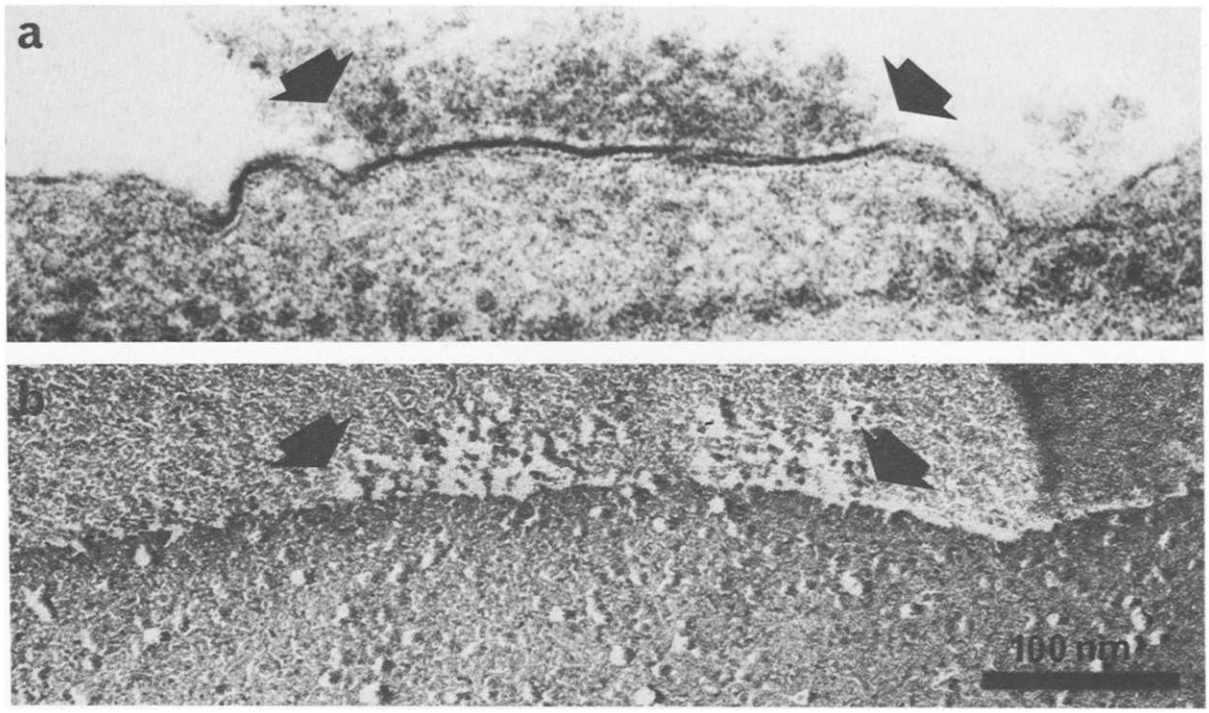

Fig. 1. Labelling of surface immunoglobulin on rabbit B lymphocytes, labelled with a horse anti rabbit-Ig antibody + FITC-conjugated anti horse-IgG antibody; magnification 258 500×; bar represents 100 nm. (a) thin section, tannic acid/glutaraldehyde fixation; (b) protoplasmic fracture face, glutaraldehyde fixation, 30% glycerol as a cryoprotectant. Arrows indicate surface label.

ture faces, i.e., a convex protoplasmic fracture face (Fig. 1b) and a concave exoplasmic fracture face (Figs. 2–5). Both fracture faces can readily be recognized by the presence of intramembraneous particles. The surrounding medium has a smooth appearance. The analogon of the label observed in thin sections can be seen in freeze-fracture electron microscopy as patches of amorphous granular material in the smooth surrounding medium, closely apposed to the edge of the fracture face of the plasma membrane (Fig. 1b, arrows). The reliability and specificity of this method have been carefully checked for different cell surface molecules and will be described elsewhere (unpublished data).

It is well-known that B lymphocytes readily redistribute their sIg upon anti-Ig treatment [10,22]. This results in 'patching' of sIg-anti Ig antibody complexes and (if energy is present and at physiological temperatures) 'capping' of these complexes [9,18]. Thereafter the complexes are cleared from the surface mainly by endocytosis [10].

When aggregation of cell surface molecules proceeds, a pattern of labelled areas becomes visible in the replica faintly shimmering through the fracture face of the plasma membrane (Fig. 2). In some instances endocytotic structures can be seen (Fig. 3) as plasma membrane invaginations coated by sIg-anti Ig complexes and accompanied by small vesicles in contact with or nearby the plasma membrane. The patterns of highly aggregated label and endocytotic structures are generally found under 'capping' conditions (i.e., energy present and at physiological temperatures). If the energy supply of the cells has been blocked by the addition of 0.1% NaN_3, such endocytotic structures are not observed and the label is found in the configuration of discrete patches. These findings closely agree with our earlier reported results using immunofluorescence techniques [9,18].

For the study of a relation of intramembraneous particles with surface immunoglobulin receptors, the sIg was labelled and redistributed under non-capping conditions (0.1% NaN_3 pre-

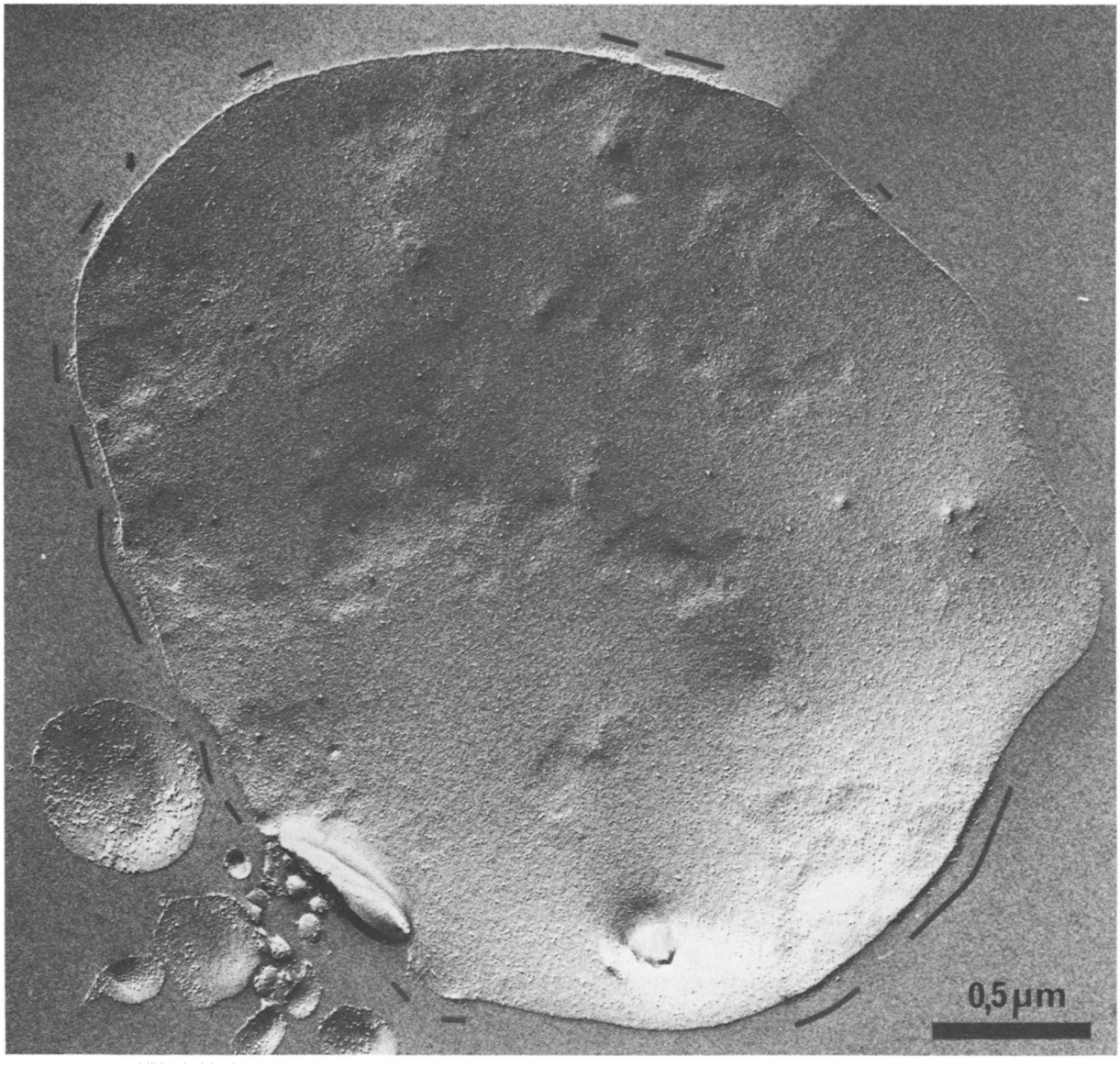

Fig. 2. Freeze-fracture replica of labelled mouse B lymphocyte, showing compact aggregates of anti-Ig label, shimmering as a pattern through the fracture face. The label itself is only visible on those sites where the pattern reaches the edge of the fracture face (indicated by line pieces). Exoplasmic fracture face, magnification 45600×; bar represents 500 nm.

sent). This resulted in the formation of sIg-anti Ig antibody patches, observed in immunofluorescence microscopy, thin-section electron microscopy (Fig. 1a) and freeze-fracture electron microscopy (Fig. 1b). In protoplasmic fracture faces we could not find any redistribution of intramembraneous particles associated with surface label. However, in external fracture faces at rather low shadow angles aggregation of small intramembraneous particles could be registered specifically coincident with the localization of sIg receptors both on rabbit (Fig. 4) and mouse (Fig. 5) B lymphocytes. Areas of the exoplasmic fracture face, where no sIg label is visible at the edge of the fracture face, have a relatively smooth appearance. These findings suggest that in areas where sIg molecules are

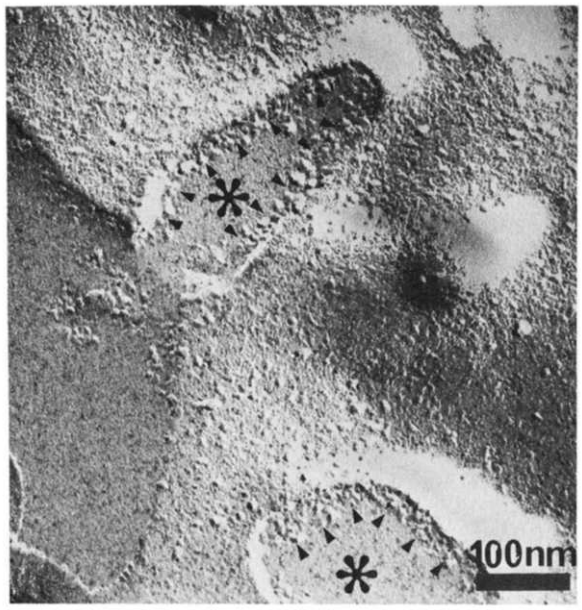

Fig. 3. Endocytosis of surface label by mouse B lymphocyte, kept at 37°C under 'capping' conditions. Invaginations of the plasma membrane can be seen (asterisks), coated by surface label (arrow heads). Exoplasmic fracture face, magnification 124080×; bar represents 100 nm.

present in high density (in patches) transmembrane molecules protrude the plasma membrane giving rise to these very small intramembraneous particles on the exoplasmic fracture face. We interpret this as a reflection of the transmembrane structure of sIg as predicted from biochemical studies [12–14].

In addition to the fact that small intramembraneous particles on the exoplasmic fracture face associated with sIg label molecules could be more readily detected in mouse than in rabbit B cells (explained by the presence of considerably more sIg molecules on murine B cells), we have the impression that a more qualitative difference between the two species can be observed. In general, intramembraneous particles in question are often found hardly protruding the lipid background of the fracture face in rabbits (Fig. 4c), while in the mouse these small intramembraneous particles can be more easily discriminated from background granulation (Fig. 5). However, we cannot make conclusions with respect to species differences of sIg insertion in the plasma membrane as image formation by Pt shadow casting at this level of resolution is still unclear.

Discussion

The possible role of intramembraneous particles as reflections of membrane proteins uncovered by freeze-fracturing has already frequently been assessed. The classical approach is by co-redistribution studies with ligands conjugated to macromolecules of well-known morphology (e.g. ferritin). In general, intramembraneous particles in the plasma membranes of eukaryotic cells did not show any co-aggregation with surface molecules [23–28]. Only in one paper [29] has a co-redistribution been claimed for intramembraneous particles and receptors for phytohaemagglutinin. However, redistributions of intramembraneous particles presented in that paper might well be explained from other effects than specific co-redistribution of intramembraneous particles and surface receptors [19,30].

Recently, Kuby and Wofsy [31] have challenged the problem by freeze-fracturing mouse lymphocytes under conditions that the majority of their membrane proteins had been redistributed into caps. They did not use a direct labelling method, but reasoned that, if intramembraneous particles would reflect transmembrane structures of these proteins, some alteration in intramembraneous particle density should be observed, dependent on whether the cell membrane had been fractured through a cap area or not. They did not observe any significant alteration of intramembraneous particle density or distribution in the protoplasmic fracture face. Intramembraneous particles in the exoplasmic fracture face decreased in density and were found in aggregates upon redistribution of surface proteins. Nevertheless, from their results they concluded that intramembraneous particles 'may not comprise a representative reflection of lymphocyte transmembrane protein molecules'.

We have challenged this problem again and in particular for two reasons: first, not all membrane-spanning proteins have to form well-defined particles of 6–12 nm diameter. For instance, glycophorin, an intrinsic protein of the erythrocyte membrane, recombined with pure lipid gives rise to ill-defined particles of 3–6 nm diameter [32,33].

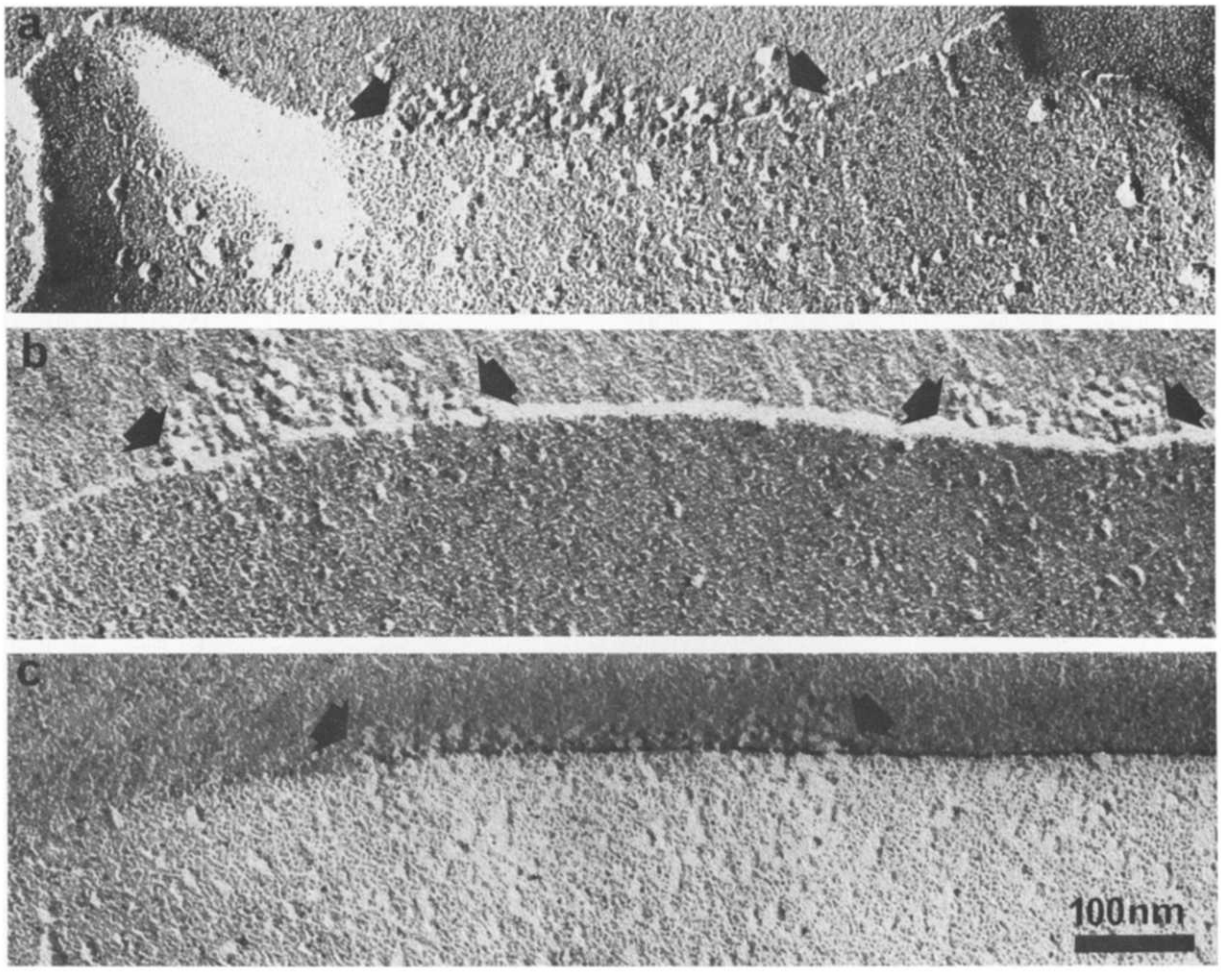

Fig. 4. Freeze-fracture replicas of rabbit B lymphocytes. Cells were labelled for 5 min at 37°C (0.1% NaN_3 present) with fluorescein-conjugated horse anti rabbit Ig antibody and subsequently with rhodamine-conjugated anti horse-IgG antibody. Note the coincidence of label at the outside of the cell (arrows) and clustering of intramembraneous particles in the fracture face hardly protruding the relatively smooth background of the membrane matrix. External fracture faces. Magnification 159600×; bar represents 100 nm.

Second, it has been suggested that during fracturing glycophorin is pulled to the exoplasmic fracture face predominantly [34]. Most studies presented so far are limited to intramembraneous particles of the protoplasmic fracture face and this is well comprehensible, as the most frequently used technique in this field (ferritin labelling and deep-etching) is only applicable to protoplasmic fracture faces and not to exoplasmic fracture faces. Only in indirect correlative studies of Matter and Bonnet [25] and of Kuby and Wofsy [31] external fracture faces were considered and only the latter report at least some effect.

We have tried to investigate the role of intramembraneous particles on both protoplasmic and exoplasmic fracture face as transmembrane structures of sIg. This was accomplished by a newly developed method, employing cells sandwich-labelled with fluorochrome-conjugated antibodies, fixed in glutaraldehyde and frozen in 35% glycerol as a cryoprotectant. With this labelling system and working under optimal freeze-etching

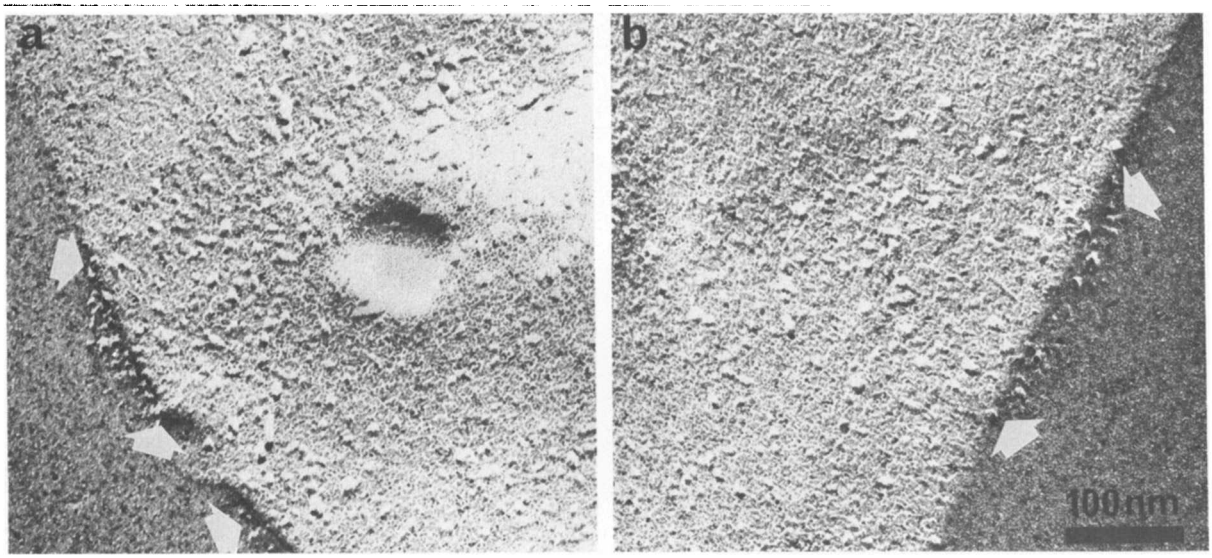

Fig. 5. Freeze-fracture replicas of mouse B lymphocytes. Cells were labelled for 5 min at 37°C (0.1% NaN_3 present) with rabbit anti mouse Ig antibody and fluorescein-conjugated horse-anti rabbit Ig antibody. Surface label is present at the fracture edge (arrows) adjacent to small fields of intramembraneous particle aggregates in the fracture face. External fracture faces. Magnification 156240×; bar represents 100 nm.

conditions ($p \leq 10^{-7}$ mbar) we were able to show aggregation of small (3–6 nm diameter) intramembraneous particles, specifically associated with sIg label. This was found in the exoplasmic fracture face, but not in the protoplasmic fracture face. Moreover, although some larger intramembraneous particles were found in the aggregates on the exoplasmic fracture face, their overall-distribution was independent of the position of sIg molecules (Fig. 5). We therefore suggest that the smaller type of intramembraneous particles involved in these sIg-associated aggregates reflect part of the membrane-spanning 'extra' peptide reported for sIg molecules [12–14].

The present findings provide an explanation for the negative results of other investigators and stress the importance of observing both protoplasmic and exoplasmic fracture face in freeze-fracture electron microscopy. On the other hand, the results reported here need some refinement. For instance, it is known that F_c-receptors of B lymphocytes may co-redistribute with sIg in rabbits [35] and in mice [36]. Moreover, differences in redistribution behaviour of surface immunoglobulins of different isotypes have been reported [37]. This might reflect a different mode of insertion in the plasma membrane. Studies are now under way to unravel the role of membrane molecules associated with sIg and the differential effect of isotype specific antisera.

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References

1 Warner, N.L. (1974) Adv. Immunol. 19, 67–216
2 Goodman, J.W. (1977) in B and T Cells in Immune Recognition (Loor, F. and Roelants, G.E., eds.), pp. 191–210, John Wiley, London
3 Melcher, U. Eidel, L. and Uhr, J.W. (1975) Nature 258, 443–445
4 Parkhouse, R.M.E., Lifter, J.L. and Choi, Y.S. (1979) in B Lymphocytes in the Immune Response (Cooper, M., Mosier, D.E., Scher, I. and Vitetta, E.S., eds.), pp. 33–38, Elsevier/North Holland, New York
5 Bourguignon, L.Y.W. and Singer, S.J. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5031–5035
6 Braun, J., Fujiwara, K., Pollard, T.D. and Unanue, E.R. (1978) J. Cell Biol. 79, 409–418

7 Flanagan, J. and Koch, G.L.E. (1978) Nature 273, 278–281
8 Gabbiani, G., Chaponnier, C., Zumbe, A. and Vassali, P. (1977) Nature 269, 697–698
9 De Groot, C., Wormmeester, J. and Mangnus-Smet, C. (1981) Eur. J. Cell Biol. 25, 202–211
10 Schreiner, G.F. and Unanue, E.R. (1976) Adv. Immunol. 24, 38–166
11 Schreiner, G.F., Fujiwara, K., Pollard, T.D. and Unanue, E.R. (1977) J. Exp. Med. 145, 1393–1398
12 Rogers, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L. and Wall, R. (1980) Cell 20, 303–312
13 McCune, J.M., Fu, S.M., Blobel, G. and Kunkel, H.G. (1981) J. Exp. Med. 153, 1684–1689
14 Dildrop, R. and Bayreuther, K. (1981) Nature 292, 61–63
15 Pinto da Silva, P. and Nicolson, G.L. (1974) Biochim. Biophys. Acta 363, 311–319
16 De Groot, C. and Leene, W. (1981) Immunobiology 159, 5–6
17 Leene, W. and Kapsenberg, M.L. (1981) in Cell Biological Aspects of Disease (Daems, W.T. et al., eds.), pp. 17–25, Nijhoff, The Hague
18 De Groot, C. and Wormmeester, J. (1981) J. Cell Biol. 24, 9–15
19 De Groot, C. and Leene, W. (1979) Eur. J. Cell Biol. 19, 19–25
20 Mühlethaler, K., Hauenstein, W. and Moor, H. (1973) in Freeze-etching. Techniques and Applications (Benedetti, E.L. and Favard, P., eds.), pp. 101–106, Societé Française de Microscopie Electronique, Paris
21 Roholl, P.J.M., Leene, W., Kapsenberg, M.L. and Vos, J.G. (1981) J. Immunol. Methods 42, 285–289
22 De Groot, C. (1980) Thesis, Amsterdam
23 Kapsenberg, M.L., Hogenes, T. and Leene, W. (1980) Proc. Dutch Fed. Meeting 21, 218 a
24 Karnovsky, M.J. and Unanue, E.R. (1973) Fed. Proc. 32, 55–59
25 Matter, A. and Bonnet, C. (1974) Eur. J. Immunol. 4, 704–707
26 Pinto da Silva, P. and Martinez-Palomo, A. (1974) Nature 249, 170–171
27 Pinto da Silva, P. and Martinez-Palomo, A. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 572–576
28 Yahara, I. and Edelman, G.M. (1975) Exp. Cell Res. 91, 125–142
29 Loor, F. (1973) Eur. J. Immunol. 3, 112–116
30 McIntyre, J.A., Gilula, N.B. and Karnovsky, M.J. (1974) J. Cell Biol. 60, 192–203
31 Kuby, J.M. and Wofsy, L. (1981) J. Cell Biol. 88, 591–598
32 Van Zoelen, E.J.J. Verkleij, A.J., Zwaal, R.F. and Van Deenen, L.L.M. (1978) Eur. J. Biochem. 86, 539–546
33 Gerritsen, W.J., Henricks, P.A.J., De Kruijff, B. and Van Deenen, L.L.M. (1980) Biochim. Biophys. Acta 600, 607–619
34 Edwards, H.H., Mueller, T.J. and Morrison, M. (1979) Science 203, 1343–1345
35 Bast, E.J.E.G., Manten-Slingerland, R., Roholl, P.J.M., Van Graft, M. and Ballieux, R.E. (1980) Eur. J. Immunol. 10, 198–202
36 Krammar, P.H. and Pernis, B. (1976) Scand. J. Immunol. 5, 199–204
37 Knapp, W., Bolhuis, R.L.H., Rádl, J. and Hijmans, W. (1973) J. Immunol. 111, 1295–1298