

CRYOPROTECTANT-INDUCED REDISTRIBUTION OF INTRAMEMBRANOUS PARTICLES IN MOUSE LYMPHOCYTES

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ABSTRACT

This study demonstrates, by freeze fracture, clustering of intramembranous particles caused by cryoprotectant treatment of intact unfixed mouse lymphoid cells. Both T and B cells react in a similar fashion, while similar clustering of particles is not observed in some other cell types. The intramembranous particles can be aggregated by incubating unfixed cells in glycerol or dimethylsulfoxide (DMSO) before freezing. The aggregation phenomenon is dependent on the length of time of exposure and the concentration of the cryoprotectants. Further, the cells remain viable and the cryoprotectant-induced clustering is completely reversible. Prefixation of glycerol-treated cells with glutaraldehyde prevents the formation of these particle clusters, and unfixed nonglycerinated cells show no clusters. Thin sections of cells exposed to glycerol or DMSO without previous fixation exhibit bizarre membrane alterations and numerous other degenerative changes. These observations stress the importance of prefixation of lymphoid cells before exposure to glycerol or DMSO, as well as indicate that the membrane characteristics of certain cell types may be probed by glycerol treatment of unfixed cells.

INTRODUCTION

During recent years freeze fracturing has become an important investigative technique for the membrane biologist. In freeze fracturing membranes are cleaved through their hydrophobic phospholipid interior (1), exposing two intramembranous fracture faces: the convex inner fracture face (A face) adjacent to the cytoplasm, and the concave outer fracture face (B face) adjacent to the extracellular space. Both fracture faces are partially covered with globular particles of variable sizes which are usually found distributed more densely on the A face.

Recent studies have shown a relationship be-

tween cell surface receptors and antigens and the distribution of the intramembranous particles (2, 3). Further, a number of investigators have shown a correlation between the number of intramembranous particles and the metabolic activity of the cell (4-6). Indeed, it has recently been proposed that at least one particular cell type can be identified by its characteristic distributional pattern of intramembranous particles (7). The plasma membranes of thymus-derived lymphocytes (T cells) have been reported to contain an intramembranous marker consisting of discrete clusters of intramembranous particles (7). Moreover, it was

proposed that for cells within the thymus, the number and size of these aggregates may be related to the differential development of this lymphocyte class.

Freezing cells without cryoprotection results in the rapid formation of large ice crystals and subsequent gross cell damage. Consequently, to insure good morphological preservation of cells, they are usually subjected to pretreatment with a cryoprotectant before freezing. It is therefore important to understand what effects, if any, cryoprotectants have on the morphology and function of cells before data from frozen preparations can be correctly interpreted.

In the present studies, we have examined the effects of two cryoprotectants, glycerol and dimethylsulfoxide (DMSO), on several subpopulations of mouse lymphoid cells. In all instances, before exposure to these cryoprotectants, we found it necessary to pretreat the cells with conventional fixatives to avoid inducing: (a) morphologic alterations in the membrane systems of the cells and (b) a rearrangement or "clustering" of the intramembranous particles within numerous lymphoid cells. Thus, data obtained using unfixed cryoprotected material should be interpreted with caution.

MATERIALS AND METHODS

Cells

Lymphoid cells from 2- to 4-mo old male C57/BL mice (Jackson Laboratories, Bar Harbor, Maine) were harvested from spleen, thymus, and bone marrow by conventional procedures using sterile techniques. Neonatal spleen cells were obtained from A/St mice (West Seneca Laboratories, Buffalo, N. Y.) less than 4-h old. In some experiments fractionated spleen cells from 24-h Mishell-Dutton suspension cultures were used. At this culture interval, most of the erythrocyte precursors present at culture initiation have died, leaving a relatively clean lymphocyte-macrophage preparation. By placing these cultures on a simple discontinuous fetal calf serum gradient, the cell aggregates (clusters) which had formed in this system settled into the lower fractions leaving a top fraction of single cells, 97% of which were lymphocytes. This top fraction of cells subsequently was observed to contain all the immune reactivity of the cultures (8).

Handling of Cells for Freeze Fracture

In all experiments cells were maintained at 0°C through all manipulations. After isolation the cells

were washed two times in Hanks' solution after a 10 s exposure to distilled H₂O for erythrocyte lysis. After washing, cells were (a) returned to fresh culture media (unfixed and unglycerinated), (b) fixed for 10 min in 1% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4, rinsed in buffer, and resuspended in a 25% glycerol solution in 0.1 M cacodylate buffer for 2 h, or (c) placed directly (i.e., unfixed) in the 25% glycerol solution, soaked for up to 2 h, then fixed as above in 1% glutaraldehyde, and returned to glycerol for an additional 2 h. (It was established that after cryoprotectant treatment of nonprefixed cells, the changes attributable to the glycerol were observed irrespective of whether the last fixation step was performed before freezing.) In one experiment DMSO was substituted for glycerol. Experiments designed to test the effects of various concentrations of glycerol were performed by substituting 5, 15, and 50% glycerol concentrations for the usual 25%. In the reversibility experiments, cells were exposed to glycerol for 2 h followed by a slowly titrated return to the culture media and, after an additional 2 h incubation, fixed in glutaraldehyde and returned to glycerol as described above.

After treatment, cells were centrifuged, the supernates were discarded, and the cell pellets were transferred to Balzers specimen holders (Balzers High Vacuum Corp., Santa Ana, Calif.), rapidly frozen in Freon 22 cooled with liquid nitrogen, and stored in liquid nitrogen. The specimens were cleaved (some receiving no etching, others etched for 2 min) in a Balzers high vacuum freeze etch unit BA 360 M, and shadowed with platinum at an angle of 45°. The replicas were strengthened by evaporation of carbon at a 90° angle, thawed, and the cells were digested from the replica by Chlorox. The replicas were mounted on 200-mesh grids and examined with a Philips 200 electron microscope. All micrographs appear with the shadowing from below.

Conventional Thin Sections

After a 2 h exposure to the cryoprotectant, cells were fixed in 1% glutaraldehyde, 0.1 M cacodylate buffer pH 7.4 for 15–30 min at 0°C. The cells were washed twice with buffer and postfixed in 1% OsO₄-water at 0°C for 60 min. The cells were then dehydrated and embedded by conventional methods in Epon 812. Thin sections were cut and stained with uranyl acetate and lead citrate.

RESULTS

Ultrastructure and Distribution of Intramembranous Particles

The intramembranous particles of mouse lymphocytes, like most other cell types, have, on freeze

fracture, a preferential association with the A face. The normal distribution of these particles on the A face is illustrated in Fig. 1. This particular arrangement of particles was seen in cell preparations which either had received no fixation and no

cryoprotection or a very brief exposure to fixation in a glutaraldehyde-cacodylate buffer mixture before cryoprotection. Particles bound to the B face, although occurring less frequently than those on the A face, exhibited a similar spatial distribution

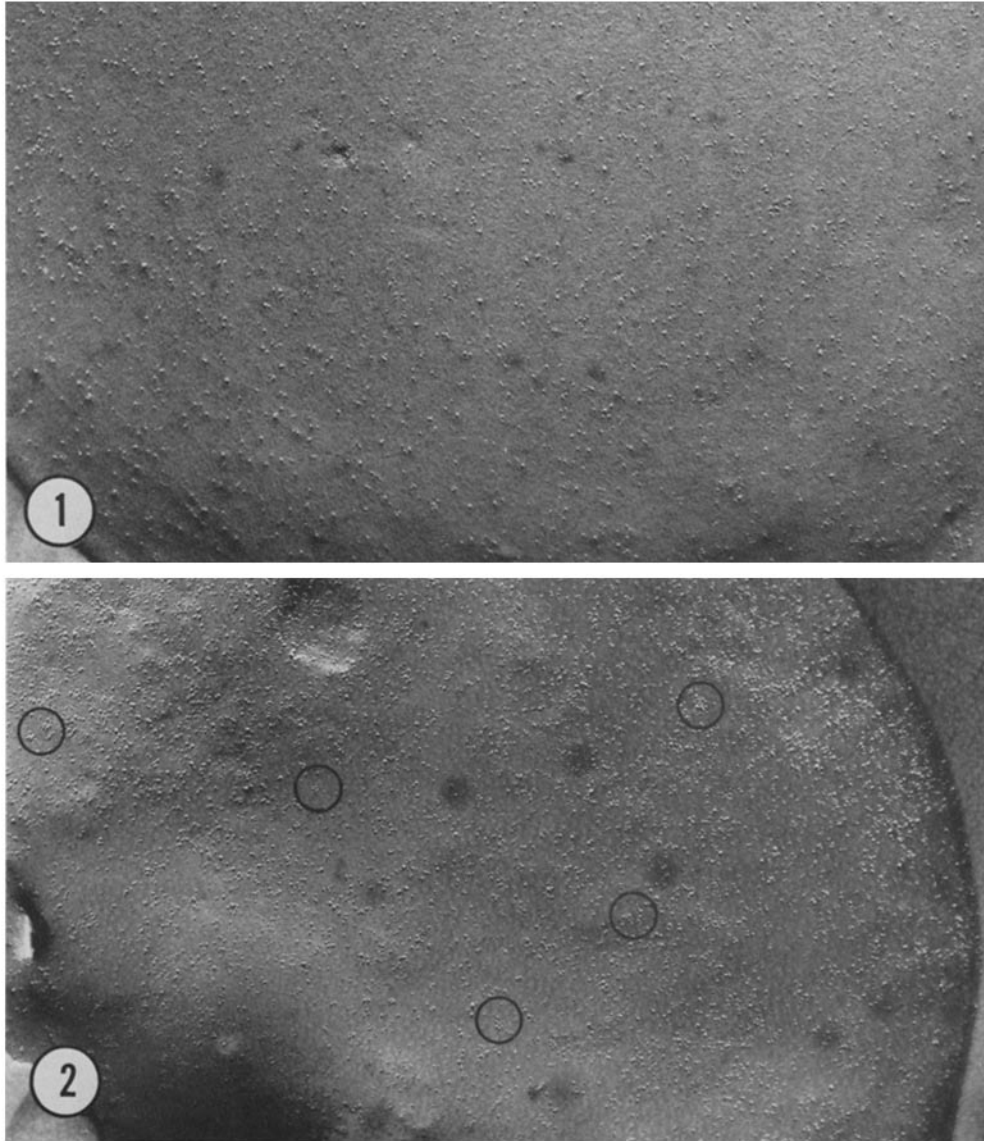


FIGURE 1 Freeze-fracture micrograph (A face) of splenic lymphocyte after 24 h in culture. This cell was prefixed lightly in 1% glutaraldehyde before exposure to glycerol. Note the monogranular distribution of the intramembranous particles. $\times 29,000$.

FIGURE 2 Freeze-fracture micrograph (A face) of splenic lymphocyte after 24 h in culture. This cell was placed in glycerol for 2 h, subsequently fixed, and returned to glycerol for freeze fracture. Note that the intramembranous particles in many areas are clustered into small aggregates of five to 10 particles. $\times 28,000$.

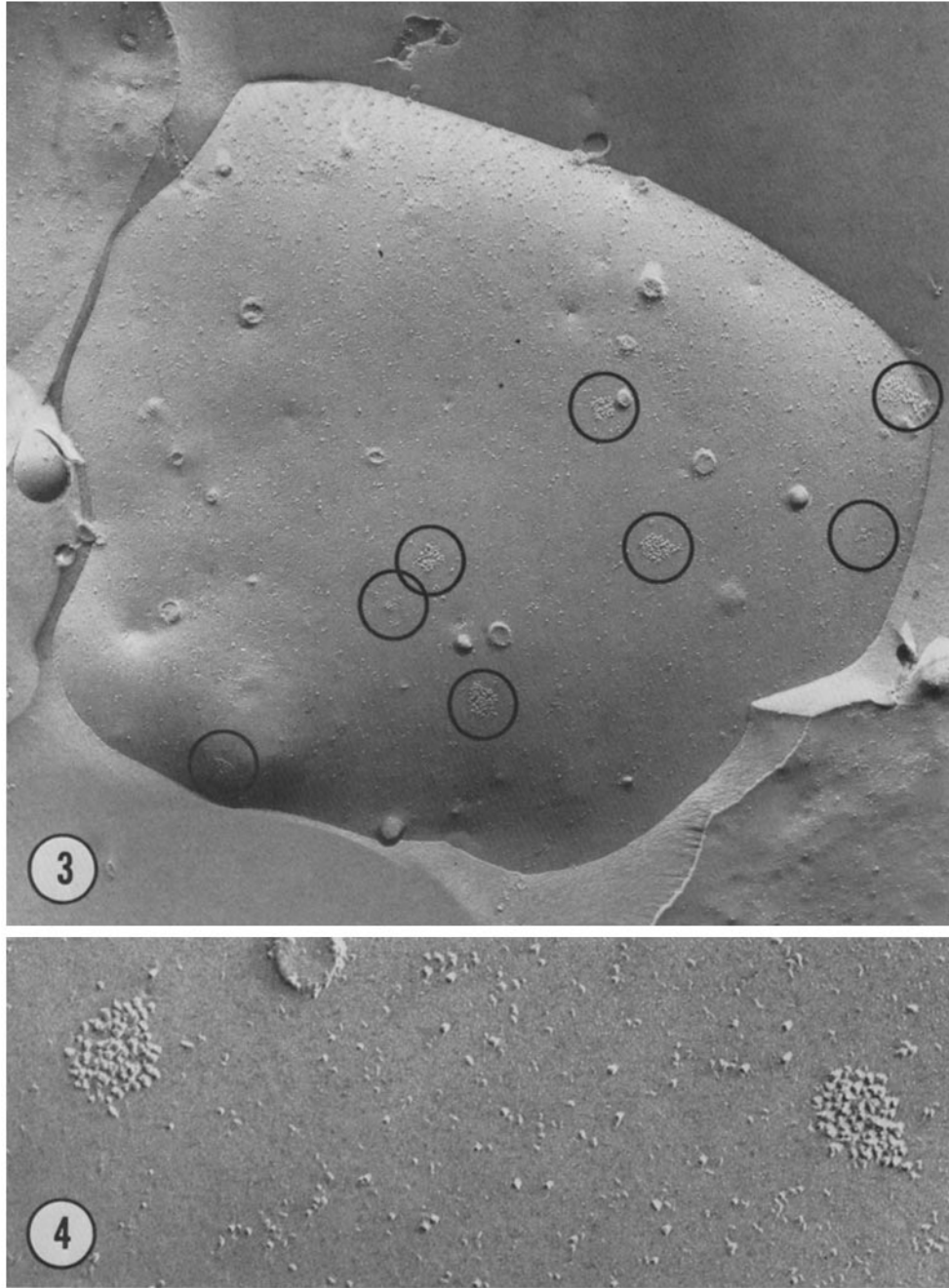


FIGURE 3 Freeze-fracture micrograph (A face) of splenic lymphocyte after 24 h in culture. This cell was soaked in glycerol 2 h, fixed in glutaraldehyde, and returned to glycerol for freeze fracture. Note how many intramembranous particles have aggregated into clusters of more than 10 particles. $\times 42,000$.

FIGURE 4 High magnification of particle clusters seen in Fig. 3. $\times 101,000$.

under these experimental conditions. A considerable number of these cells, however, if placed in glycerol or DMSO without a prefixation step will experience a "rearrangement" of their intramembranous particles to form discrete clusters of particles. While the clustering is easier to detect on the particle-rich A face, careful examination of B faces has shown that to an equal degree a similar situation exists (9). On the basis of their distribution, these clusters of particles have been divided arbitrarily into two groups (7): (a) small clusters of five to 10 particles (Fig. 2) and (b) large clusters, which have more than 10 particles (Fig. 3). Fig. 4 shows at high power the distribution of particles within the large clusters. Monogranular distribution refers to single particles or clusters of less than five particles. Particle dimensions were determined to be $85 \pm 2 \text{ \AA}$ (standard error of the mean) irrespective of whether the particles existed singularly or within clusters.

Kinetics of Particle Aggregation in Nonprefixated Cryoprotected Cells

These studies were done using cells isolated from the top fraction of gradients run on 24-h spleen cell cultures since differential cell counts using ultrastructural criteria had shown the majority of cells present in this fraction to be lymphocytes (8). Fig. 5 illustrates the rapidity by which incubation in 25% glycerol induces the formation of particle clusters in unfixed mouse lymphoid cells maintained at 0°C. Note that the clustering of intra-

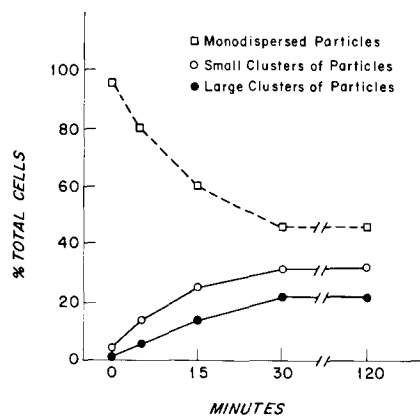


FIGURE 5 Effect of time on the formation of clusters of intramembranous particles in splenic lymphocytes cultured for 24 h. Within 30 min at 0°C maximal clustering has occurred. Each point represents an average of three counts of 100 cells.

membranous particles was well underway during the initial 5 min exposure and that 30 min was sufficient for glycerol to exert its maximal effect. Additional overnight incubation did not significantly alter the 30-min determinations.

Cell Permeability

Cell viability was determined by trypan blue exclusion (10) and indicated that approximately 90% of the cells remained viable in the cryoprotectant during their 2 h exposure (Table I). Thus, the rearrangement or clustering of intramembranous particles was not related to an increased permeability of the cells to the dye.

Effect of Glycerol Concentration

Clustering of intramembranous particles in cultured spleen cells was observed to be markedly influenced by the concentration of glycerol used for cryoprotection (Fig. 6). With the usual concentration of 25%, approximately half the unfixed cells were involved in particle clustering after 2 h exposure. In glycerol concentrations ranging from 5 to 50% the number of unfixed cells exhibiting clustered particles increased from virtually 0 to 70%.

In one experiment, unfixed cells were not subjected to glycerol but were pelleted and frozen in normal culture media. While this resulted in the formation of huge ice crystals and gross cell damage, numerous small areas of membrane could be examined. It is important to note that no clustering of particles was observed in any of these cells and no change in the A/B face distribution of particles occurred.

TABLE I
Permeability of Cryoprotected Cells to Trypan Blue*

Exposure time to glycerol	Increase in dye permeability
min	%
0	0
0 + H ₂ O	2‡
1	4
5	6
15	9
120	10

* Spleen cells taken from 24-h cultures (see Materials and Methods).

‡ Loss of viability due to exposing the cells to H₂O for erythrocyte lysis.

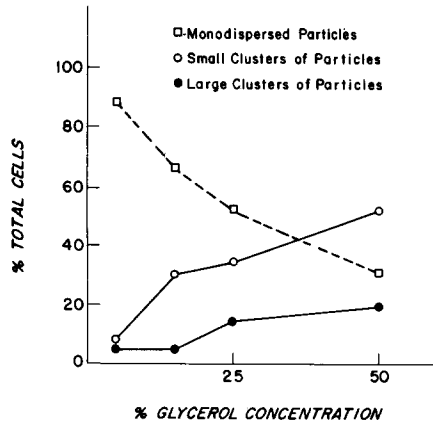


FIGURE 6 Effect of the concentration of glycerol on the number of intramembranous particle clusters seen in splenic lymphocytes after 24 h culture. Each point represents an average of three counts of 100 cells in two similar experiments.

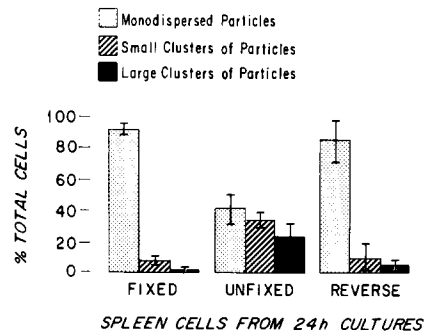


FIGURE 7 Reversibility of clustering of intramembranous particles in splenic lymphocytes after 24 h culture. Cells in Fixed column were subjected to 10 min fixation in 1% glutaraldehyde before exposure to glycerol. Cells in Unfixed column were placed directly into 25% glycerol, soaked for 2 h, then fixed in glutaraldehyde before return to glycerol. Cells in Reverse column were treated as unfixed for the first 2 h but were subsequently returned to their original culture media for an additional 2 h before glutaraldehyde fixation and a second exposure to glycerol. The bars represent standard deviations from four counts of two experiments.

Reversibility of Clustering

Since we established that the cryoprotectants could induce intramembranous particle clustering, we questioned next the permanence of this effect. Unfixed cultured spleen cells which had been exposed to 25% glycerol for 2 h were slowly titrated back into normal culture media. This procedure diluted the glycerol to a final concentration of less

than 1% in the fresh culture media. Subsequently, the cells were incubated in this new mixture for 2 h, then fixed in glutaraldehyde, and returned to glycerol before freezing. Fig. 7 shows that glycerol-induced particle aggregation is completely reversible.

Cells from Other Lymphoid Compartments

Intramembranous particles in cells taken from mouse thymus, bone marrow, and spleen were also seen to form clusters when exposed to 25% glycerol for 2 h without prefixation (Figs. 8 and 9). Very little difference was observed between the total number of cells exhibiting clusters in each tissue;

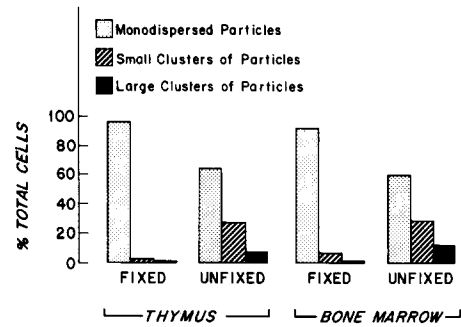


FIGURE 8 Effect of glutaraldehyde fixation on the aggregation of intramembranous particles in mouse thymic and bone marrow lymphocytes. Clearly, the particle clustering effect of glycerol can be expressed to an equal extent in both of these lymphoid compartments.

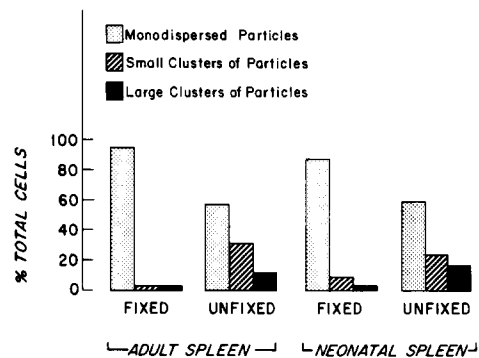


FIGURE 9 Effect of glutaraldehyde and glycerol on two different populations of mouse splenic lymphocytes. In both experiments the number of lymphoid cells exhibiting particle clusters was similar. Since the neonatal spleen does not contain functional T cells, this observation supports the contention that glycerol can produce particle clustering in all lymphocyte subclasses.

approximately 40% for thymus, bone marrow, and spleen, as opposed to 58% for cultured spleen cells. This difference is most likely explained by the presence of nonlymphocytic cells, whose intramembranous particles do not appear susceptible to redistribution by glycerol.

Since this particular clustering of intramembranous particles has been reported to occur only in T lymphocytes (7) and because our data showed such high proportions of lymphocyte involvement, we were inclined to believe that it was a property of lymphocytes in general. To examine this possibility, we next pooled spleen cells from neonatal mice which have been reported to lack detectable T cell function (11). This experiment (Fig. 9) showed that the intramembranous particles in numerous cells in the neonatal mouse spleen (which presumably have not yet been populated by thymus lymphocytes) were susceptible to the effects of glycerol. In addition, two experimental procedures known to enrich the number of mature T cells were done to further eliminate the possibility that intramembranous particle clusters are intrinsic to mature T cells. First, cortisone-resistant thymocytes were prepared according to the method of Mosier and Pierce (12). Second, spleen cells (educated T cells) were obtained from lethally irradiated, thymus-reconstituted mice, subsequently injected with sheep erythrocytes. In both instances glutaraldehyde prefixation of these lymphoid cell populations prevented the formation of particle aggregates.

Ultrastructural Changes in Cryoprotected Cells

The most apparent ultrastructural changes seen in thin sections of glycerol-treated cells were associated with the membrane systems of the cell (Fig. 10). The nuclear envelope appears swollen and distended and often several profiles of laminated membrane reside inside these spaces. The mitochondria show various signs of degeneration; some appear normal while others have disintegrated cristae and are greatly swollen. The Golgi region is quite expansive and is a prominent feature within these cells. Most noticeable are the numerous myelin-like figures dispersed throughout the cytoplasm. Rarely does a section not pass through one or more of these structures. Unlike the DMSO-treated cells, however, the vesicles in glycerol-treated cells seldom appear associated with the cell surface. With the exception of myelin

figures similar glycerol-induced membrane changes have been observed in HeLa cells (13).

Although both glycerol and DMSO cause changes in cell structure, it is apparent from Fig. 11 that DMSO produces more dramatic changes. When unfixed cells are subjected to DMSO, not only do the intramembranous particles cluster, but numerous membrane blisters appear. These blisters are always devoid of intramembranous particles and, when viewed in thin sections (Fig. 12), appear as numerous vesicles located on the cell periphery. Unexpectedly, this membrane blistering occurred even after glutaraldehyde prefixation but to a lesser extent. In general, the cell morphology looks very poor with most of the cell organelles showing some degree of disruption. The nuclear membrane appears fragmented, the mitochondrial cristae are disrupted, and the ribosomes form loose aggregates leaving some barren areas within the ground cytoplasm. Many vesicles are surrounded by small electron-opaque particles (probably ribosomes) and numerous lysosome-like dense bodies are located throughout the cytoplasmic matrix.

Nonlymphoid cells

Nonlymphoid cells studied included macrophages, thymus epithelial cells, erythrocytes, and toad bladder epithelium. Unlike lymphocytes, these cells did not show cryoprotectant-induced clustering of intramembranous particles.

DISCUSSION

This study has demonstrated that the intramembranous particles of murine lymphoid cells can be clustered by exposing unfixed cells to glycerol or DMSO. This clustering was shown to be reversible, preventable by glutaraldehyde prefixation, and not unique to any one subpopulation of lymphocytes. Particle aggregation occurred within minutes after exposure to glycerol and maximal clustering was observed by 30 min. Also, the total number of cells exhibiting clusters was found to be dependent upon the concentration of glycerol used. Further, without the glutaraldehyde prefixation, both glycerol and DMSO were responsible for numerous perturbations in conventional cellular fine structure.

A recent observation linking the clustering of intramembranous particles to a specific lymphocyte subclass (mature T cells) has been reported by Mandel (7). He found a marked reduction of

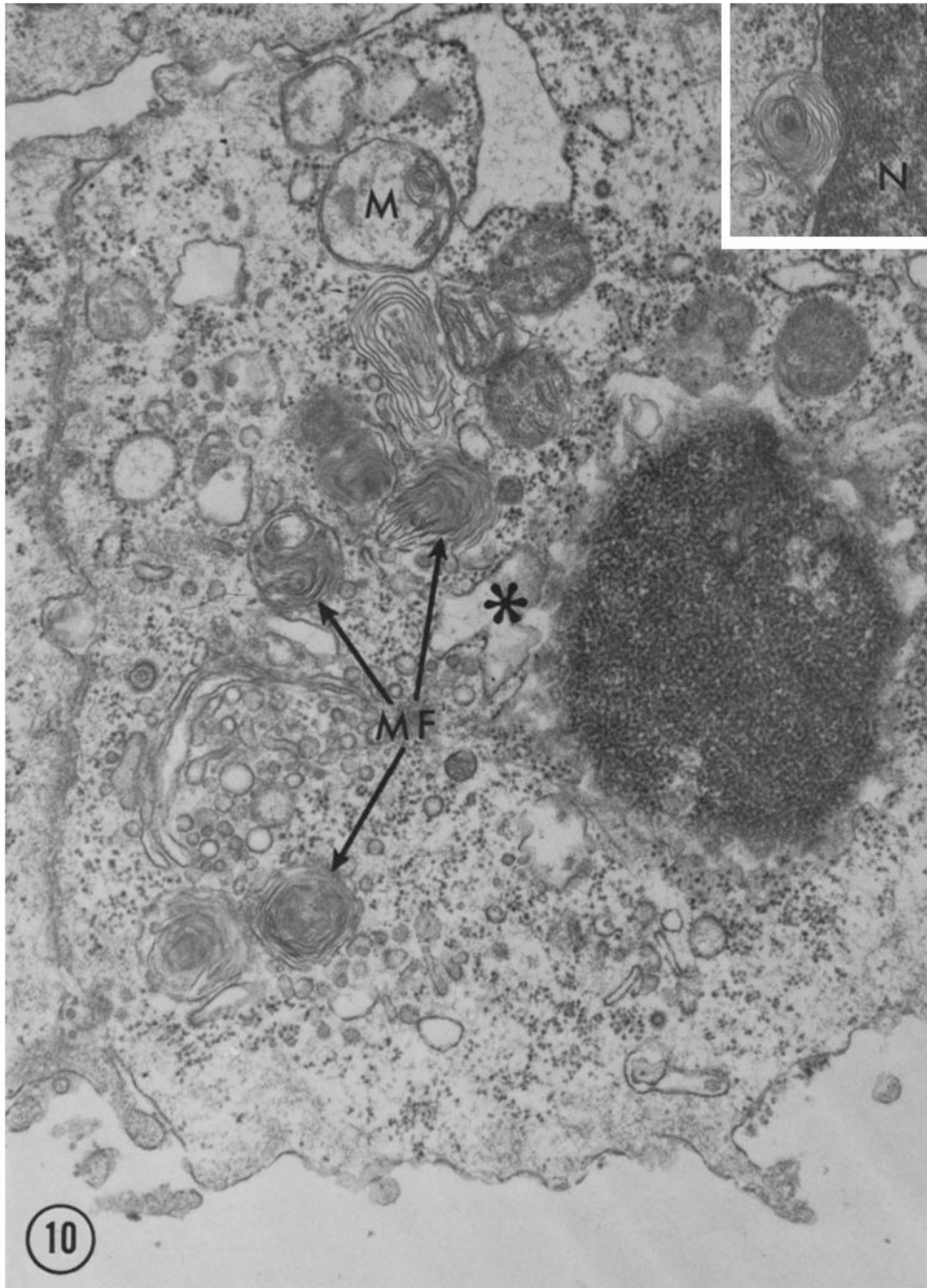


FIGURE 10 Electron micrograph showing the effect of glycerol on unfixed lymphocytes. Changes appear largely limited to the membrane systems within the cell. Numerous myelin-like figures (*MF*) are present. The nuclear envelope (*) is swollen and distorted. Some mitochondria (*M*) appear to be disintegrating. $\times 30,000$. *Inset*: a myelin-like figure within the nuclear membrane of a similar cell type. *N*, nucleus. $\times 28,000$.

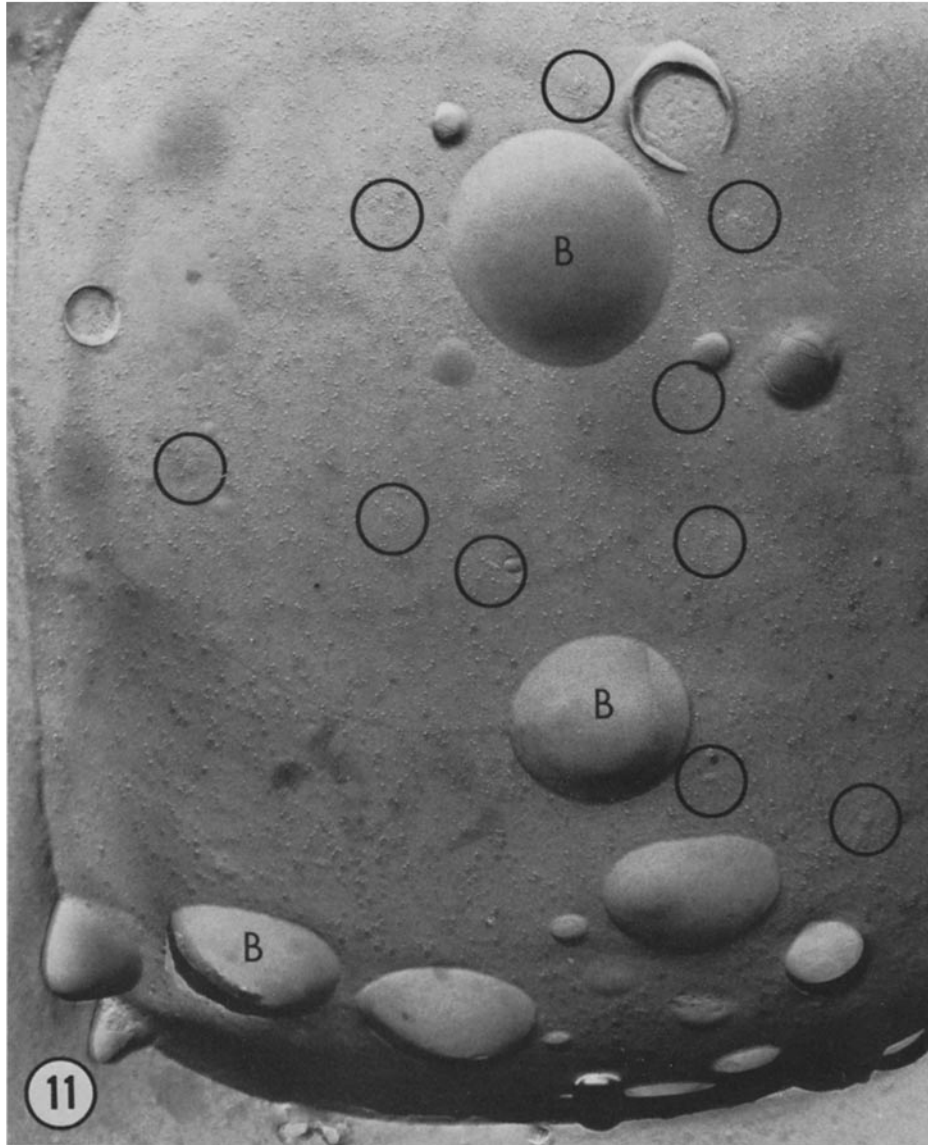


FIGURE 11 Freeze-fracture micrograph (A face) illustrating that DMSO not only causes intramembraneous particle aggregation in unfixed mouse splenic lymphocytes, but in addition it can induce the formation of large membrane blisters (*B*) all of which are devoid of intramembraneous particles. $\times 39,000$.

lymphocytes exhibiting clusters when lymphoid cells were obtained from T cell-depleted or thymic-deficient mice. Furthermore, according to his interpretation, the degree of particle clustering represented various stages in the functional maturity of this lymphocyte class.

Our data are inconsistent with these conclusions for a number of different reasons. First, Mandel did not prefix his cells before exposing them to

glycerol. Therefore, it is highly probable that he observed the same cryoprotectant-induced particle clustering that we report in the present studies. Second, the particle clusters we observed in unfixed neonatal spleen cells (which reportedly lack T cell functions [11]) strongly suggest that it is the B cell which is affected in this system, not the T cell. Third, we found that by exposing lymphocytes to several different concentrations of glycerol, we

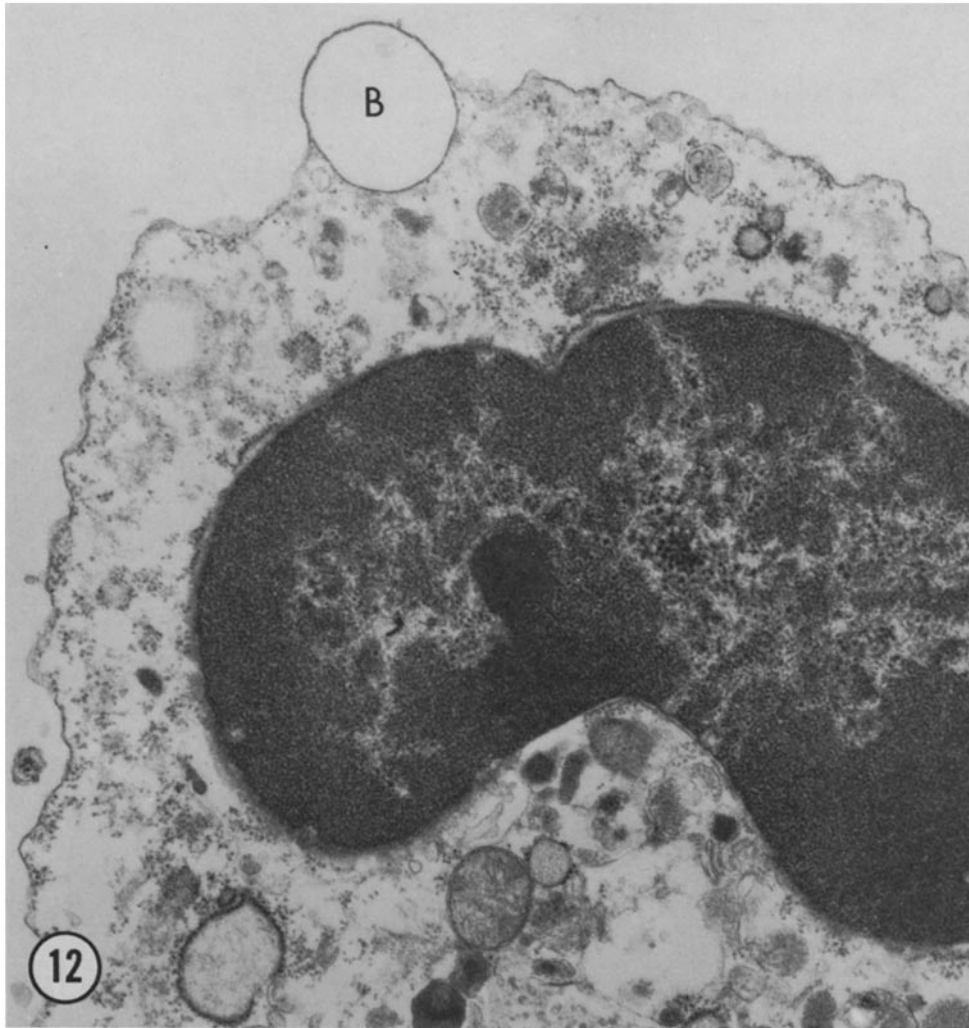


FIGURE 12 Conventional electron micrograph showing a cross-sectional view through a blister (B) similar to those seen in Fig. 11. Note also that exposure of unfixed cells to DMSO results in aggregation of ribosomes and extraction of ground cytoplasm. $\times 29,000$.

could regulate the number of polygranular cells present. This was especially noteworthy since previous studies (8) using fluorescent antibody techniques to analyze our experimental system had shown that: (a) 50% of the lymphocytes present had surface immunoglobulins (a B cell marker) and (b) 30% of the lymphocytes contained the θ antigen (a T cell marker). Despite the low percentage of θ -positive cells, high concentrations of glycerol could induce particle clustering in 70% of the total lymphocytes present. Clearly, to obtain this high percentage both B and T cells must be affected. Finally, in experiments

designed to increase the mature T cell population, cells taken from the thymus of cortisone-treated mice and spleen cells obtained from lethally irradiated, thymus-reconstituted mice subsequently injected with sheep erythrocytes (educated T cells) showed less than 1% particle aggregation when prefixed in glutaraldehyde before placement in a cryoprotectant. Thus, we can conclude that intramembranous particles can be induced to cluster in either T or B lymphocytes whenever these cells are exposed to glycerol without prefixation.

Worthy of note is the observation that even with

glutaraldehyde prefixation, a small percentage of lymphoid cells in our studies exhibited particle clusters (Figs. 5-9). What this means is not entirely clear. The possibility that these cells represent a distinct subpopulation of lymphocytes seems remote since the unfixed unglycerinated cells never contained particle clusters. Perhaps the brief exposure of cells to such low concentrations of glutaraldehyde in our system is not sufficient to completely inhibit the particle clustering effect of the cryoprotectant.

The clustering phenomenon we report here is outwardly similar to experiments in which, at low pH, reversible clustering of intramembranous particles was demonstrated in erythrocyte ghost membranes, but not in intact cells (14). Glutaraldehyde fixation prevented clustering and particle aggregation occurred at low temperatures. A recent report (15) suggests that for particles to be aggregated at low pH, three major peptides (two spectrin components and an unknown smaller peptide) were released into the supernate while preparing or pretreating erythrocyte ghosts. Whether glycerol or DMSO, being able to penetrate intact lymphocyte membranes, may cause particle clustering by a similar mechanism is at present unknown.

Clustering of intramembranous particles was also observed by Branton et al. (15) in their studies on the organism *Acholeplasma laidlawii*. When the growth medium was enriched with straight chain fatty acids, some particle aggregation was noted, which was enhanced at lower temperatures. The authors suggested that, in the case of saturated fatty acid-fed organisms, the physicochemical properties of the membrane allow for close packing and association of membrane lipids with concomitant displacement of the intramembranous proteins. Conceivably, a similar mechanism is operative in lymphocytes treated with glycerol and DMSO in that these substances may cause a rearrangement of lipids in the membrane leading to the exclusion of intramembranous particles in certain areas and aggregation in others.

The freeze-fracture morphology of unfixed glycerol-treated cells was normal with the exception of the clustered intramembranous particles. In sharp contrast, unfixed DMSO-treated, freeze-fractured cells, in addition to exhibiting clustered intramembranous particles, had numerous membrane blisters which were devoid of intramembranous particles. Both B and A faces, were populated with blisters devoid of particles. Conse-

quently, the most plausible explanation for this phenomenon would be to envisage a DMSO-induced bubble produced within the hydrophobic region of the membrane. The actual cleavage properties within these bubbled membranes are quite complex and will be examined in depth in a separate communication.

In conclusion, these studies demonstrate that lymphocyte membranes have the peculiar property of being susceptible to cryoprotectant-induced aggregation of intramembranous particles. Both T and B lymphocytes undergo this rearrangement. Particle aggregation is reversible, and is prevented by glutaraldehyde fixation. Finally, particle movement occurs at low temperature in viable intact cells. Although glycerol treatment of unfixed lymphocytes changes the inherent distribution of particles, such treatment may prove to be a useful probe for delineating certain properties of lymphocytes and possibly other cell membranes.

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