*Biochimica et Biophysica Acta*, 600 (1980) 590—594


## BBA Report

BBA 71477

# FREEZE-FRACTURE OF RECONSTITUTED MODEL MEMBRANES USED AS TARGETS FOR CELL-MEDIATED CYTOTOXICITY

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(Received March 31st, 1980)

*Key words: Transplantation antigen; Freeze-fracture electron microscopy; Cytotoxicity; Reconstitution; Model membrane; (T-cell)*

## Summary

In a recent communication (Hollander, N., Mehdi, S.Q., Weissman, I.L., McConnell, H.M. and Kriss, J.P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4042—4045) we reported that reconstituted model membranes containing murine tumor cell membrane proteins can be substituted for living cells as targets for cell-mediated cytolysis by allosensitized T-lymphocytes. The specificity of the lytic process was governed by the appropriate histocompatibility antigen (H-2). It was stressed, however, that although a standard protocol was faithfully followed for the reconstitution of the target membrane vesicle, the system was not uniformly reproducible. Some experiments showed high levels of specific vesicle killing while no lysis was observed in others. This work extends our description of the structural requirements of reconstituted membrane vesicles.

EL4 (H-$2^b$) tumor cells were maintained in ascites form by intraperitoneal inoculation of C57B1/6 mice. Eye muscle tissue was obtained post mortem. Effector cells were generated in mixed lymphocyte culture (Balb/c spleen cells sensitized to C57B1/6 spleen cells) as described previously [1]. Plasma membranes were prepared from cells or tissue as described in detail [1]. Solubilization of membrane proteins and their insertion into model

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membranes was by modifications of the procedure described for the insertion of thryotropin receptors into lipid vesicles [2]. Briefly, sonicated lipid vesicles (5 mg dimyristoyl phosphatidylcholine, 10 mg dipalmitoyl phosphatidylcholine and 3 mg cholesterol) were mixed with detergent-treated EL4 (5 mg) and eye muscle (5 mg) membrane proteins, layered over a discontinuous sucrose gradient consisting of 15 ml 50% sucrose and 15 ml 15% sucrose in phosphate-buffered saline, pH 8.2, and centrifuged at 95 000 × *g* for 24 h. Two distinct bands of reconstituted membranes were obtained. The heavier band at the interface of the two sucrose solutions (band I) contained 55—65% protein, while a lighter band 2—3 mm above it contained 5—20% protein (band II). The two bands were carefully removed by aspiration and separately dialysed against phosphate- buffered saline, pH 7.4, for 14—20 h. They were then pelleted by centrifugation at 10 000 × *g* for 20 min. Each pellet was resuspended in 0.5 ml phosphate-buffered saline, pH 7.4, containing 1 mCi of $Na^{51}CrO_4$ and sonicated to optical clarity under nitrogen using a bath sonicator (approx. 10 min). Free $^{51}Cr$ was removed by dialysis against excess phosphate-buffered saline, pH 7.4, overnight and finally against RPMI 1640 for 1 h. Vesicles were counted in a hemocytometer and used as targets for sensitized lymphocytes by the standard assay procedure [1]. The cytotoxic efficiency of the effector cells was determined by using $^{51}Cr$-labeled EL4 cells as targets. The value for total dialysable radioactivity was obtained from vesicles treated with 5% Triton X-100. The percentage of specific vesicle lysis was calculated as follows:

$$\frac{^{51}Cr \text{ released in test} - {}^{51}Cr \text{ released in control}}{^{51}Cr \text{ released with detergent}} \times 100$$

where $^{51}Cr$ released from vesicles alone without any additions served as control.

Results presented in Table I show that vesicles prepared from reconstituted membranes of band I (Fig. 1a) are specifically lysed by lymphocytes sensitized to $H\text{-}2^b$ but not by those sensitized to $H\text{-}2^d$. In this experiment, vesicle lysis was highest at an effector: vesicle ratio of 20:1 and lysis was also observed at other ratios. In several experiments, specific vesicle lysis was obtained only at a ratio of 20:1, none at lower ratios and a protective effect was observed at higher ratios. Vesicles prepared from reconstituted membranes of band II (Fig. 1b) have never been used successfully as targets for sensitized lymphocytes.

Vesicles used as targets in the experiments described above were examined by freeze-etch electron microscopy (Fig. 1). Small drops of vesicle suspensions (0.5-1.0 μl) were pipetted onto 3-mm diameter copper planchets resting on a metal block at room temperature. After 2—3 min equilibration, the planchets were plunged for 5 s into partially liquified Freon 22 at liquid $N_2$ temperature. The frozen samples were then transferred to liquid $N_2$ for storage. Samples were fractured and etched at —106° C in a Balzers BAF 301 Freeze-Etching Device equipped with electron beam apparatus for platinum and carbon shadowing and a quartz-crystal monitor for regulating platinum and carbon film thickness. The etching time was 45 s, and platinum film

TABLE I

SPECIFIC $^{51}Cr$ RELEASE FROM TARGET VESICLES AT 6 h

| Lymphocytes | Effector: vesicle ratio | % $^{51}Cr$ released from vesicles containing EL4 (H-2$^b$) membrane proteins | |
|---|---|---|---|
| | | Band I | Band II |
| C57B1/6 (H-2$^b$) unsensitized | 100:1, 50:1, 20:1, 1:1 | 0.5 ± 1.5 | 2 ± 2 |
| C57B1/6 (H-2$^b$) sensitized to Balb/c (H-2$^d$) | as above | 0.5 ± 2.5 | 1 ± 3 |
| Balb/c (H-2$^d$) unsensitized | as above | 0.5 ± 1.5 | −1 ± 3 |
| Balb/c (H-2$^d$) sensitized to C57B1/6 (H-2$^b$) | 100:1 | 9 | 3 |
| | 50:1 | 18 | 3 |
| | 20:1 | 23 | −1 |
| | 1:1 | 6 | 0 |

Lymphocytes allosensitized in mixed lymphocyte culture and unsensitized lymphocytes were incubated with $1 \times 10^5$ vesicles prepared from reconstituted membranes of band I and band II as described in detail in the text. Specific $^{51}Cr$ release was measured at 6 h. Control release was of the order of 5—10% of specific release.

deposition was approx. 20 Å. After shadowing, the replicas were floated from the planchets onto water and transferred to a sodium hypochlorite solution for 2—3 h. They were then rinsed on water, transferred to 85% ethanol, and picked up on 400 mesh copper grids. Electron microscopy was performed with a Hitachi HU-11E instrument at a magnification of 57 000. Micrographs were taken on 3¼ × 4 in Kodak film.

Fig. 1 shows freeze-etch electron micrographs of several different vesicle preparations. Fig. 1a is a representative micrograph of a vesicle from band I. The membrane surface has a very lumpy texture, and fracture faces along the bilayer midplane are rarely seen in vesicles from this band. Moreover, vesicles from this band frequently cast very pronounced shadows during the freeze-etch process, as shown in Fig. 1a. This suggests that membranes in these vesicles are very rigid. Lumpy features similar to some of those seen in Fig. 1a have been observed previously in a number of well-defined, reconstituted, lipid-protein membranes (see, e.g., Refs. 3 and 4), but the protein: lipid ratio in these reconstituted systems is often not as high as that found here. In Fig. 1b a representative vesicle from band II is shown. Vesicles from this band have smooth surfaces that are typical of lipid bilayer membranes without protein. Fracture faces along the bilayer midplane are frequently observed in the freeze-etch replicas of these vesicles.

Vesicles prepared from band I were routinely used as targets for cell-mediated lysis. In most of these experiments there was significant lysis. In a few preparations, vesicles from band I were not successfully lysed by sensitized lymphocytes. In these instances, freeze-etch electron microscopy showed vesicles that were smooth, with only occasional lumps (Fig. 1c), and fracture faces along the bilayer midplane were observed frequently. When radio-immunoassay was used to compare the H-2$^b$ contents of several dif-

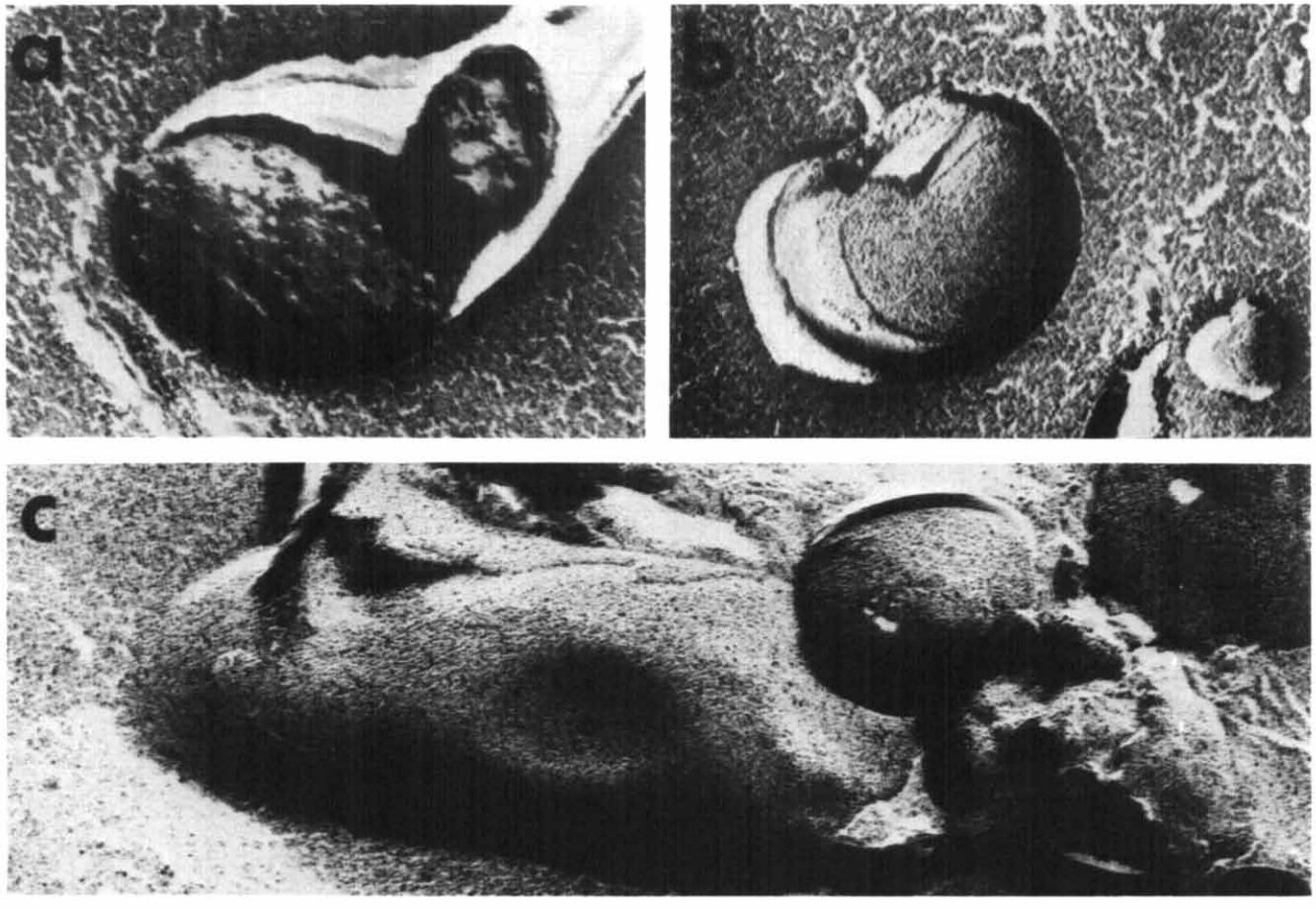


Fig. 1. Freeze-etch electron micrographs of membrane vesicles reconstituted from bands I and II. (a) Vesicle from a preparation of band I in which high specific lysis was obtained. (b) Vesicle from band II. (c) Vesicle from a preparation of band I in which low specific lysis was obtained. Magnification, 142 000 X.

ferent preparations of vesicles from band I (anti H-$2^b$ serum was obtained from Dr. H.O. McDevitt), it was found that vesicles from preparations that gave high specific lysis (i.e., Fig. 1a) contained approx. 6—10 times more antigen than vesicle preparations that gave low specific lysis (i.e., Fig. 1c). A further complication in all vesicle lysis experiments arose from the contamination of large (1—2 $\mu$m) vesicles from band I with variable amounts of limit-size vesicles (not shown). These small vesicles are always smooth as determined by freeze-etch studies. Since the presence of large numbers of these vesicles appeared to contribute to the failure of several experiments, attempts were made to separate them from the large vesicles. Gel filtration through Sepharose 2B (Pharmacia, Uppsala, Sweden) and sucrose density gradient centrifugation proved unsuccessful because the limit-size vesicles were found to adhere to the large vesicles.

On the basis of the experimental results described above, it appears that a minimum structural requirement for vesicle lysis is a lumpy texture of the type exhibited in Fig. 1a. Such a texture probably reflects the high density of proteins in the vesicle membranes used here. It is noteworthy that this protein:lipid ratio of approx. 60:40 is similar to the ratio for the natural plasma membrane. There may also be a minimum vesicle size requirement for vesicle lysis; however, it is likely that the lack of vesicle lysis in very small vesicles is simply related to the smooth texture of these vesicles.

This work has been supported by the National Institutes of Health Grant No. 5R01 AI13587 and the National Science Foundation Grant PCM 77-23586. J.T.L. has been supported by the Cellular and Molecular Biology Training Grant GM7276 from the National Institutes of Health.

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