Immunogenicity of Cytolipin H Aggregated with *Acholeplasma laidlawii* Membrane Proteins (35907)

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The list of immunologically active lipids found in membranes of normal and malignant cells is growing steadily since the isolation of cardiolipin in 1942 and of cytolipin H in 1958 (1, 2). The purified lipids usually function as haptens, failing to elicit an antibody response when injected into animals (2). Antisera to the lipid haptens have, therefore, been prepared either by injection of tissue fractions or of the purified lipids mixed with protein of a different species. Both methods are not completely satisfactory as tissue fractions may contain several serologically active lipids resulting in sera of low specificity, and the injection of the purified lipid hapten mixed with swine serum or albumin and adjuant frequently fails to elicit a good antibody response to the lipid (2).

A new approach to the preparation of antibodies to lipid haptens has recently been proposed by us (3). Purified glycolipid haptens of *Mycoplasma pneumoniae* were bound to membrane proteins of *Acholeplasma laidlawii* by a reaggregation process (4) which consisted of solubilization of lipid-depleted *A. laidlawii* membranes and *M. pneumoniae* glycolipids in 20 m*M* sodium dodecyl sulfate (SDS) followed by dialysis of the mixed solutions against 20 m*M* Mg^{2+}. The reaggregated material elicited the production in rabbits of antibodies which fixed complement and precipitated the purified glycolipids (3, 5). Free glycolipids or their mixture with *A. laidlawii* membrane proteins were far less effective in evoking these antibodies (3). The main advantage in utilizing hybrid reaggregates consisting of protein and lipid derived from membranes of two different organisms lies in the ability to select the lipid and the protein components of the reaggregate and thus control the type of antibodies produced. The reaggregation process seems to involve an intimate binding of lipid to protein, presumably by the same kind of bonds responsible for their association in biological membranes. The hybrid reaggregates prepared from mixed membrane proteins and lipids of *A. laidlawii* and *Mycoplasma gallisepticum* (6) or from membrane proteins of *A. laidlawii* and glycolipids of *M. pneumoniae* (7), contained triple-layered membranes in addition to amorphous clumps and myelin-like structures. The possibility of producing hybrid reaggregates of mycoplasma membrane proteins and lipids of animal cell membranes has not been investigated as yet. Cytolipin H (lactosyl ceramide) is a suitable candidate for this study since it is not present in microbial membranes and the available methods for producing specific and potent antisera to it are limited.

Cell membranes of *A. laidlawii* were isolated by osmotic lysis of the organisms (4) and were depleted of lipid by aqueous acetone extraction (3). The lipid-depleted membranes were solubilized by adding 1 ml of 0.1 *M* SDS solution to 4 ml of a membrane suspension that contained 35 mg of protein. Chromatographically pure cytolipin H (10 mg) isolated from bovine spleen (8) was dissolved in 0.5 ml of chloroform–methanol (2:1 by vol). Two milliliters of deionized water and 0.5 ml of 0.1 *M* SDS were added to the lipid solution. The resulting emulsion was heated under nitrogen until all the chloroform evaporated and the emulsion cleared. The cytolipin H solution was then combined with 5 ml of the membrane protein solution

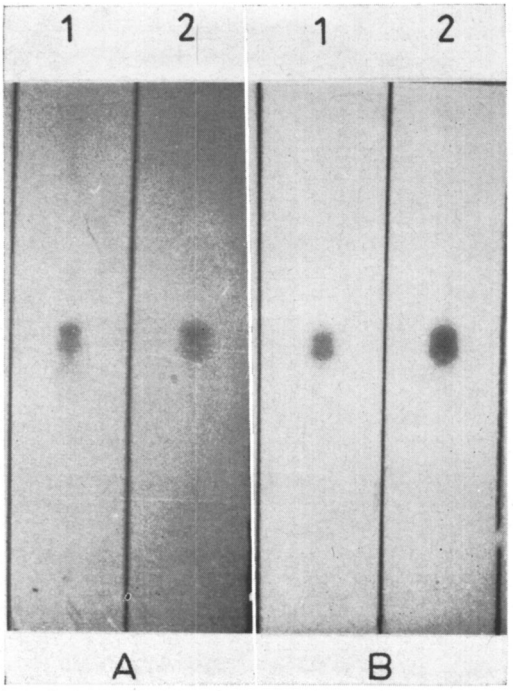

FIG. 1. Thin-layer chromatography of the lipid extracted from *A. laidlawii* membrane protein aggregated with cytolipin H: (1) lipid extract; (2) pure cytolipin H; (A) chromatogram developed with iodine vapor to show all lipid spots; (B) chromatogram sprayed with the periodate–Schiff reagent to show glycolipids (3). Cytolipin H appears as two partially separated spots caused by the separation of the cytolipin H molecules bearing $C_{16:0}$ chains from those containing the $C_{24:0}$ and $C_{22:0}$ chains (2).

in SDS and the mixture was dialyzed for 4 days in the cold without stirring against 2 liters of dilute B-buffer (4) containing 20 m*M* $MgCl_2$. The contents of the dialysis bag were centrifuged at 36,000*g* for 90 min in the cold; and the pellet was resuspended in 7 ml of deionized water. A portion (0.5 ml) of the suspension was extracted with chloroform–methanol, 2:1. Thin-layer chromatography (3) showed this extract to consist almost entirely of cytolipin H (Fig. 1) demonstrating its incorporation into the reaggregated material, and the almost complete absence from the hybrid reaggregate of other lipids. Over 80% of the solubilized cytolipin H and about 60% of the membrane proteins were found in the reaggregated material.

The suspensions used for immunization contained 0.44 mg/ml of lipid-depleted *A. laidlawii* membranes or membrane proteins aggregated with cytolipin H. Cytolipin H (0.16 mg/ml) was suspended in phosphate-buffered saline, together with 2 mg/ml of lecithin dissolved in a small volume of ethanol (3). Rabbits were injected intradermally at several sites with a mixture of 2 ml of complete Freund's adjuvant. The same volume of injected material, but mixed with incomplete adjuvant was given intradermally 3 weeks later. After an additional 3-week interval, 2 ml of the antigen was injected intraperitoneally. Sera were collected from the marginal ear vein before immunization and at 3, 6, and 7 weeks after the first injection. The rabbits were exsanguinated at the end of week 9. The sera of the immunized rabbits were inactivated (57°, 30 min) and tested for antibodies to cytolipin H by complement fixation (9). The test antigen was composed of cytolipin H combined with 100 parts by weight of lecithin–cholesterol (1:2, w/w) and suspended in saline (10). Antigen titrations were carried out using six 50% hemolytic units and 50 μl of the tested serum diluted 1:5. The antisera were classified as negative, weak, intermediate, or strong according to the antigen titer. The reactivity of antisera classified as intermediate or strong was confirmed by conducting antibody titrations with 40 ng of cytolipin H.

Table I shows that the hybrid reaggregate made of cytolipin H and *A. laidlawii* membrane proteins elicited a fairly strong antibody response to cytolipin H in essentially all of the rabbits. A response was detectable after 3 weeks. Cytolipin H by itself was a poor immunogen; of the 5 rabbits receiving it 3 did not show any antibody response and the other 2 responded only late in the immunization period. The *A. laidlawii* membrane proteins, when injected alone, did not evoke antibodies to cytolipin H; only 1 out of 6 rabbits exhibited an antibody response, and this one was weak (Table I).

Antisera reacting with cytolipin H have so far been produced with four kinds of immunizing antigens: tissue fractions (human tumor, human spleen) (9), tissue lipids added to swine serum (2), conjugated proteins

TABLE I. Antibody Response in Rabbits to Free and Aggregated Cytolipin H.

Immunizing antigen	No. of rabbits	Immunization period (weeks)	Antibody response to Cytolipin H[a] (no. of rabbits)			
			Negative	Weak	Intermediate	Strong
Cytolipin H aggregated with *A. laidlawii* membrane proteins	9[b]	P[c]	9	0	0	0
		3	0	3	5	1
		6	0	1	2	4
		7	0	0	2	5
		9	0	0	1	6
Cytolipin H alone	5	P	5	0	0	0
		3	5	0	0	0
		6	4	0	1	0
		7	4	0	0	1
		9	3	0	1	1
A. laidlawii membrane proteins alone	6	P	6	0	0	0
		3	5	1	0	0
		6	6	0	0	0
		7	5	1	0	0
		9	5	1	0	0

[a] Determined by complement fixation tests (9). Negative, weak, intermediate, and strong are based on the following titers (referred to 1 μg of cytolipin H/ml equal to 1): negative, <2; weak, 2 to 6; intermediate, 6 to 20; strong, >20.

[b] Two of the rabbits died after 3 weeks.

[c] P = preimmunization serum.

(porcine γ-globulin-azophenyl lactoside) (10), and conjugates of lactosyl sphingosine with a synthetic peptide copolymer of alanine, glutamic acid, and tyrosine (11). Immunization of rabbits with human tumor fractions frequently gives rise to anticytolipin H antibodies, but these sera may be multivalent and may react with other tissue lipids as well (9). Immunization with the globulin-azophenyl lactoside conjugate was considerably less efficient, and the percentage of anti-lactose sera that were useful reagents for the measurement of cytolipin H by complement fixation was low (10). The preparation of the synthetic polypeptide conjugate of lactosyl sphingosine is inconvenient (11). Our new method is relatively simple and is very effective in producing potent antisera to cytolipin H. *Acholeplasma laidlawii* grows rapidly and large quantities of essentially pure membranes can be easily obtained by osmotic lysis of the organism (12). Furthermore, the chances are so small that some of the *A. laidlawii* membrane proteins are serologically related to membrane proteins of animal cells that antisera to hybrid reaggregates, through containing antibodies to *A. laidlawii* membrane proteins (3), may be specific for animal cell membranes, reacting only with the cytolipin H component. The binding by reaggregation of other lipid haptens of animal origin to *A. laidlawii* membrane proteins seems feasible, and experiments are now under way to test the production of antisera to gangliosides, a very weak immunogen, by the new method.

We thank Dr. B. Prescott for his help and Mr. W. D. James for expert assistance. The work was supported in part by a grant from the National Cancer Institute CA-10903 and from the National Science Foundation (GB-12242).

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Received April 19, 1971. P.S.E.B.M., 1971, Vol. 138.