Biochimica et Biophysica Acta, 597 (1980) 216—223

BBA 78661

ANTISERUM TO THE MILK FAT GLOBULE MEMBRANE

PREPARATION AND CAPACITY TO SUPPRESS MILK SECRETION

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(Received July 17th, 1979)

Key words: Milk secretion; Exocytosis; Antiserum; Antibody; Lectin; (Mammary gland, Milk fat globule membrane)

Summary

A procedure is described for preparing rabbit antiserum to goat milk fat globule membrane. This membrane is derived from the secretory surface of the lactating cell. Immunoelectrophoresis and enzyme-linked immunosorbent assay showed that antibody development reached maximal levels in about 6—8 weeks. Infusion of 5—10 ml of this antiserum into the lactating mammary gland of goats via the teat canal depressed milk yields temporarily on the infused side to 60—80% of normal. Ordinary serum from rabbit, goat or human did not evoke such a response and rabbit complement was not essential for the effect. Fractionation showed that the globulin fraction of the antiserum contained the milk-suppressing principle. Milk from the antiserum-infused side of the udder showed extensive and tenacious clumping of fat globules on standing 12—24 h. The inhibition of milk flow by antibodies to the secretory membrane resembles a previously observed inhibition following infusion of concanavalin A or its succinyl derivative. Binding of antibodies or lectins which recognize specific surface protein components of the lactating cell appears to be involved in the suppression mechanism. The possible relevance of our findings to autoimmune suppression of exocytosis is noted.

Introduction

In efforts to reveal the molecular mechanisms of milk secretion we have been infusing various probe molecules into the lactating gland of the goat via the teat canal [1—4]. A strong suppressing effect on milk secretion was produced by

concanavalin A [2], a plant lectin which has the ability to bind specifically to cell surface glycoproteins containing glucosidic or mannosidic linkages and to cross-link with other concanavalin A so bound. Recent research [5—7] has shown that concanavalin A binds to several glycoproteins of both human and bovine milk-fat globule membrane. Because the milk-fat globule membrane is derived from the (secretory) plasma membrane of the lactating cell, we postulated that the suppression of milk secretion by concanavalin A involves its diffusion and binding to glycoproteins of the plasma membrane.

The concanavalin A-glycoprotein reaction is like an antigen-antibody reaction at a cell surface. This suggested that antibodies to the milk-fat globule membrane also might suppress milk secretion. The procedure for producing an active antiserum and its effectiveness as an inhibitor of milk secretion were investigated. A preliminary report of this work has been given [8].

Materials and Methods

Preparation of antigen

Milk fat globule membrane was prepared from fresh goat's milk as described by Dowben et al. [9]. The procedure involves release of membrane from washed globules by churning. The sedimented membrane was resuspended in 15—20 ml of 0.002 M Tris buffer, pH 7.0, first with the aid of a spatula and then using several 5-s bursts of sonication. In all, five membrane preparations were made. Recovery data on a protein basis [10] obtained for three preparations averaged 49 ± 24 mg/l of milk. Composition and properties of these preparations (data not shown) closely resembled those of bovine milk fat globule membrane (for review see Ref. 11).

Antiserum production

The membrane suspension (antigen) was injected intradermally at multiple sites over the shaved back and sides of rabbits according to the technique described by Campbell et al. [12]. At least 20 sites per injection were used. On a protein basis 1 or 5 mg of antigen in 1 ml was injected. The initial injections were made using antigen emulsified in Freund's complete adjuvant in a 1 : 3 ratio. For subsequent injections antigen was emulsified with incomplete Freund's adjuvant. Each animal was bled once before immunization and the collected serum was used as a control serum (normal rabbit serum). An initial injection of antigen was made using the multiple site technique. On the 10th day after injection whole blood was collected via the central ear artery. On the 14th day freshly prepared antigen was again injected with bleeding again 10 days later, etc. Three injections of antigen at the 5-mg level were required to produce readily detectable levels of antibodies by immunoelectrophoresis. Collected blood was allowed to clot for 2 h, cooled to 4° C and held at that temperature for 12—20 h. The serum was decanted into 15-ml conical centrifuge tubes and centrifuged at 1600 rev./min for 10 min to sediment any cells. The serum was transferred to vials and held at —20° C until needed.

Characterization of the serum: The enzyme-linked immunosorbent assay technique [13] was used to evaluate the serum from each rabbit bleeding regarding an increase in antibodies (titer) against the antigen. The assay proce-

dure was conducted in disposable polystyrene microtiter plates [14] using horseradish peroxidase conjugated to goat antirabbit IgG as the marker enzyme [15] for the amount of antibody bound to the antigen. It was reacted with specific substrate (H_2O_2 + 5-aminosalicyclic acid) resulting in color development which was measured spectrophotometrically at 440 nm. Calculations based on absorbance were used to evaluate development of antibodies in rabbit serum.

The appearance of antibodies in the rabbit serum was also monitored by conventional immunoelectrophoresis procedures [16]. For this purpose a Gelman electrophoresis chamber (Gelman Instrument Co., Ann Arbor, MI) and agar-coated glass slides (25 × 75 mm) were used.

The globulin fraction of 5- to 10-ml quantities of antiserum active in suppressing milk secretion was isolated by exhaustive dialysis against distilled water at 2°C. Dialyzing-tubing with a cut-off of 12 000 molecular weight was used. The precipitated globulin in the dialysate was sedimented by centrifugation (Sorvall Model RC5B) at 10 000 × *g* for 30 min. The protein pellet was dispersed by agitation in 5 ml of 0.9% aqueous KCl solution. The procedure was estimated to yield a serum globulin preparation of 80—90% purity with albumin as the major contaminant. The globulin solution was infused in the manner of the antisera to determine its effect on milk yield.

Infusion of antisera into lactating goats and analysis of milkings

Goats on a normal hay-grain ration and yielding 300 to 900 ml of milk per udder half in 12 h were used to test the effects of milk fat globule membrane antiserum on secretion and composition of milk. The goats were milked at 12 h ± 15 min intervals and the quantity of milk from each gland was recorded. Several milkings were made prior to infusion of antiserum to establish the baseline condition. Antisera, 3—10 ml, were infused using a syringe connected to a cannula (1.5 mm internal diameter) inserted into the teat canal. These infusions were made immediately following a milking so a 12-h period existed during which the gland was filling and the antiserum could react throughout the tissue. Portions of each milking were used for fat and protein determinations and for microscopic observations of fat globule aggregation. Fat globule clumping was observed by diluting the milk 1 : 3 with 0.15 M sodium cacodylate buffer (pH 7.1) and viewing under a cover slip at 100 and 400 × magnification. Fat content was determined by Milko-Tester (turbidimetry) and milk protein by Pro-Milk Automatic Analyzer (dye-binding) (both instruments by Foss Electric Co., Hillerod, Denmark).

Results

Immunoelectrophoresis revealed three major antibody bands induced in the rabbit sera by milk fat globule membrane-antigen, Fig. 1. No bands developed with control sera. The two antisera of Fig. 1 were collected after six injections of 5 mg of antigen according to the previously described schedule. The progessive increase of antibody titer over time using this schedule is shown in Fig. 2.

Rabbit sera with antibodies as shown (weeks 10—16) in Fig. 1 suppressed milk yield when infused into the mammary gland of the lactating goat, Fig. 3.

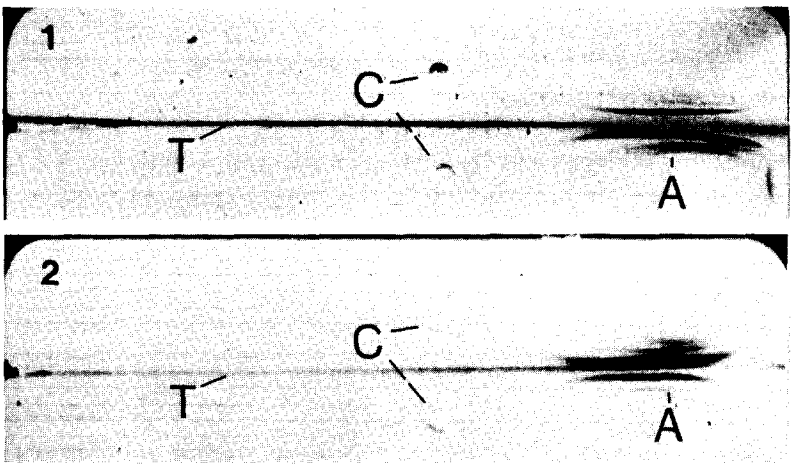

Fig. 1. Immunoelectrophoresis of two rabbit antisera (1 and 2) showing antibodies (A) to goat milk fat globule membrane. Antisera in the center wells (C) were subjected to electrophoresis in barbital buffer containing 1% agar, ionic strength 0.025 and pH 8.8. Milk fat globule membrane (MFGM), 5 mg/ml on a protein basis, was placed in the troughs (T) and by its diffusion into the gels fixed positions of the antibodies.

The inhibiting effect occurred only in the infused side of the udder and was not produced by infusion of control sera. Infusions were made of sera collected after injection of antigen up to six weeks without noticeable depression in milk yield although antibodies could already be detected in some of these sera by immunoelectrophoresis. Generally at least six injections of antigen were

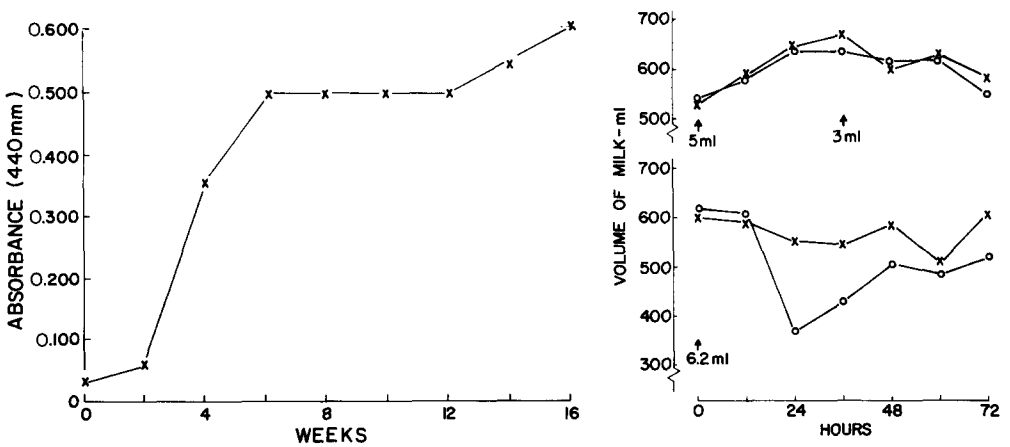

Fig. 2. The relationship between amounts of antibodies to goat milk fat globule membrane in rabbit serum and time using a biweekly program of successive injections and bleedings. Absorbance data from the ELISA procedure are an index of antibody concentration in the serum.

Fig. 3. Yields of milk in sequential 12-h milkings of a goat following intramammary infusions of rabbit serum via the teat canal. X———X, volume of milk (ml) from the uninfused side of the udder; o———o, volume from the infused side. Upper, yields following infusion of 5 and 3 ml of control serum. Lower, yields following infusion of 6.2 ml of rabbit antiserum to milk fat globule membrane. Infusions were made immediately following the indicated (↑) milkings.

required to generate an antiserum that would suppress milk yield 20—40% in at least one milking. Using this lower limit we have produced four effective antisera as tested in three different goats.

The depression in milk yields of the magnitude shown in Fig. 3 are not trivial. Actually the 12-h milkings tend to normalize and understate effects. It is quite probable that the restriction in milk flow is more extensive over some

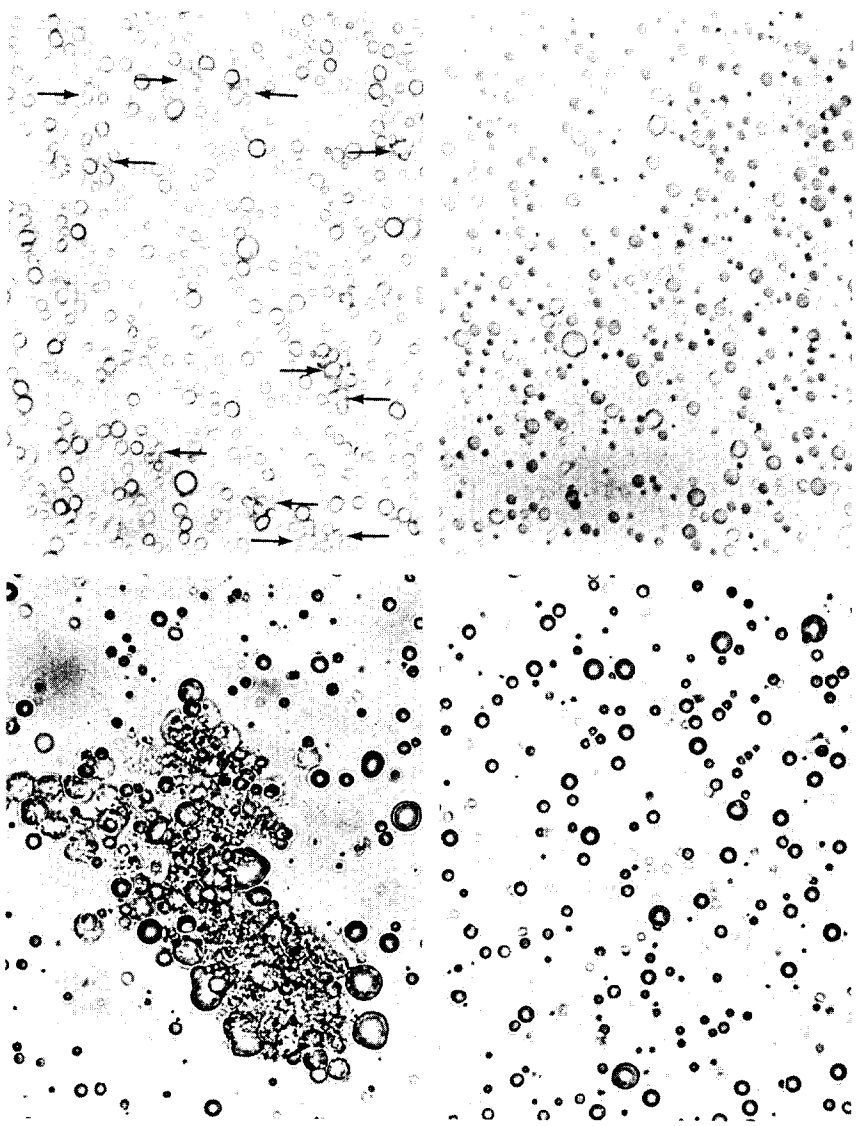

Fig. 4. Disposition of fat globules in goats milk 12 h following intramammary infusion of rabbit antiserum to goat milk fat globule membrane (5 ml). Right panel: Milk from uninfused half of udder, fresh (upper) and after 24 h storage at 4°C (lower). Left panel: Milk from half of udder infused with rabbit antiserum, fresh (upper) and aged (lower) as for the control milk. Note fat globule clumps (arrows) in milk from the infused side, relatively small with fresh milk and larger in the aged milk. Milks diluted with buffer 1 : 3, respectively. Magnification 570X.

shorter period of time. We also surmise that all of the secreted fat globules still contained in the gland in the first 12-h post-infusion milking are binding sites for milk fat globule membrane antibodies, therefore we suspect that globules in this milk substantially competed for antibodies for reaction with the plasma membrane of the cell.

Following infusion of antisera which depressed milk flow it was noticed that the next milking from that side of the udder would produce a cream layer that on standing 12—24 h could not be dispersed. Even after violent shaking many visible pieces of the cream layer could be seen, whereas normal goat's milk forms a cream layer very slowly and it can be totally dispersed by gentle shaking. Microscopic examination of the milks from the infused and uninfused sides of the udder showed fat globule clumping in the freshly drawn milk from the infused side. Such milk viewed under 400 X magnification averaged 14 globule clusters per field whereas milk from the uninfused side exhibited less than 1 cluster per field. This difference, which is shown in Fig. 4, was seen to be a consistent result of infusing high-titer antisera.

It was considered of interest to determine whether complement in the antiserum is essential for the depressing effect on milk secretion. Antiserum before and after complement inactivation by heating at 56°C for 30 min was infused into a lactating goat. The animal in question was giving about 500 ml of milk per gland per 12 h. The unheated antiserum was first infused into one side of the udder and after the milk flow returned to normal in several days the complement-inactivated serum was infused into the other side. The difference in milk yields between the infused and uninfused sides are shown in Fig. 5 together with differences in the protein contents of the milk. Complement from the rabbit serum does not appear to be a factor in the suppression of milk secretion. A temporary rise in milk protein was observed as volume of milkings decreased. No significant variations in fat contents of the milks were detected. Average values for fat content (with ranges) in the infused sides were 3.37

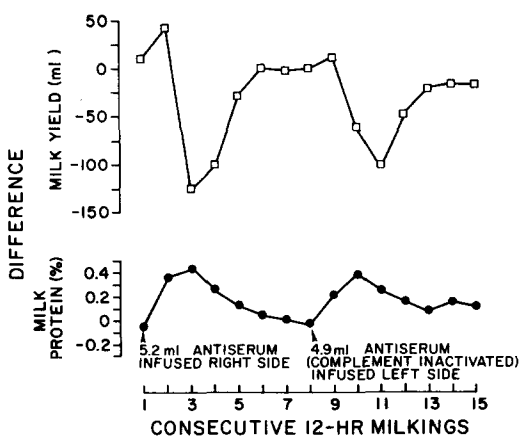

Fig. 5. Differences in yields of milk and protein contents of milkings as a result of infusing rabbit antiserum to goat milk fat globule membrane into one half of the udder of a goat. Variations in the infused side are expressed wtih the uninfused side as reference. Antisera with and without complement inactivated were infused as indicated after the first and eighth milking.

(2.96—3.54) and 3.48 (3.24—3.90)% and in the uninfused sides 3.50 (3.24—3.90) and 3.62 (3.26—3.98)%, respectively.

Infusions of the globulin fraction from antisera produced depressions in milk yield similar to those shown in Figs. 3 and 5 although the magnitude of the effect was somewhat less than with unfractionated antiserum.

Discussion

Concanavalin A, which reacts with cell surface glycoprotein, can suppress milk secretion in the lactating goat [3] and antiserum to the milk fat globule membrane acted similarly. Glycoproteins on the secretory surface of the lactating mammary cell should be effective antigenic components of the milk fat globule membrane. Functionally, the induced antibodies resemble concanavalin A as to site and mode of action in the mammary gland. One possible mechanism might involve a change in distribution of membrane proteins required for exocytosis by cross-linking the bound lectin or antibody. We recently tested succinyl concanavalin A, which binds but does not cross-link in the manner of concanavalin A [17], for its capacity to suppress milk secretion in the lactating goat. It was similar in effect to concanavalin A (Patton, S., unpublished). This suggests further experiments with Fab fragments (antigen binding fragments) prepared from milk fat globule membrane antibodies to see if they too would suppress milk secretion. Like succinyl concanavalin A these fragments bind, but do not cross-link in distinction to intact immunoglobulins [18]. Another way in which binding of antibodies or concanavalin A to the cell surface might reduce milk secretion is by interfering with recycling of plasma membrane that accumulates as a result of exocytosis.

A further consideration concerns evidence that concanavalin A receptors in the exterior of plasma membrane are somehow coupled to actin-myosin filaments on the cytoplasmic side of the membrane (see Ref. 19 and citations therein). It is conceivable that such a (sliding) filament system facilitates fusion of secretory vesicles with plasma membrane in exocytosis and that functioning of the system is inhibited by binding of concanavalin A, succinyl concanavalin A or antibodies. Evidence of structural connections (pre-exocytotic attachment plaques) between secretory vesicles and plasma membrane in lactating rat mammary cells has been presented by Franke et al. [20].

An interesting implication of this work is that foreign species sera appear to have no detrimental effects on mammary tissue or lactation in the goat. Besides repeated injections of rabbit serum we have also infused serum from other goats and on one occasion human serum. These produced no swelling or inflammation and milk yields remained normal. Even with rabbit antisera, milk flow returned to normal within a few days. This is in contrast to the effects of foreign serum on zymogen granule secretion by the mouse pancreas in which secretion by the cells is suppressed at first and then cell necrosis occurs [21].

It seems biomedically relevant that secretory activity (exocytosis) of organs and tissues may be influenced by antibodies or lectins. In addition to our observations regarding antibodies and milk flow, serum antibodies reacting with the surface membrane of pancreatic islet cells are frequently detected in insulin-dependent diabetes mellites [23]. There appears to be an important

research issue in revealing the extent and molecular nature of this type of constraint on exocytosis.

Acknowledgements

Dr. C.D. Ercegovich (deceased) and Greg Williams assisted with immunological aspects of the work. This research was made possible by Biomedical Support Grant 2192 of the U.S. Public Health Service and assistance of the Pennsylvania Agricultural Experiment Station (Journal series paper No. 5750).

References

1 Patton, S. (1976) J. Dairy Sci. 59, 1414—1419
2 Patton, S. (1976) FEBS Lett. 171, 154—156
3 Patton, S., Stemberger, B.H. and Knudson, C.M. (1977) Biochim. Biophys. Acta 499, 404—410
4 Knudson, C.M., Stemberger, B.H. and Patton, S. (1978) Cell Tissue Res. 195, 169—181
5 Keenan, T.W., Franke, W.W. and Kartenbeck, J. (1974) FEBS Lett. 44, 274—278
6 Newman, R.A. and Uhlenbruck, G.G. (1977) Eur. J. Biochem. 76, 149—155
7 Murray, L.R., Powell, K.M., Eigel, W.N. and Keenan, T.W. (1978) J. Dairy Sci. 61 (Suppl. 1), 158
8 Patton, S., Ercegovich, C.D. and Trams, E.G. (1978) J. Dairy Sci. 61 (Suppl. 1), 161
9 Dowben, R.M., Brunner, J.R. and Philpott, D.E. (1967) Biochim. Biophys. Acta 135, 1—10
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
11 Patton, S. and Keenan, T.W. (1975) Biochim. Biophys. Acta 415, 273—309
12 Campbell, D.H., Garvey, J.S., Cremer, N.E. and Sussdorf, D.H. (1970) Methods in Immunology, 2nd edn., W.A. Benjamin, New York, NY
13 Engvall, E. and Perlmann, P. (1971) Immunochemistry 8, 871—874
14 Voller, A., Bidwell, D.E., Huldt, G. and Engvall, E. (1974) Bull. World Health Org. 51, 209—211
15 Van Weemen, B.K. and Schuurs, A.H.W.M. (1971) FEBS Lett. 15, 232—236
16 Williams, C.A., Jr. and Grabar, P. (1955) J. Immunol. 74, 397—403
17 Bornens, M., Karsenti, E. and Avrameas, S. (1976) Eur. J. Biochem. 65, 61—69
18 Porter, R.R. (1959) Biochem. J. 73, 119—126
19 Condeelis, J. (1979) J. Cell Biol. 80, 751—758
20 Franke, W.W., Lüder, M.R., Kartenbeck, J., Zerban, H. and Keenan, T.W. (1976) J. Cell Biol. 69, 173—195
21 Nevalainen, T.J., Fowlie, F.E. and Janigan, D.T. (1977) Lab. Invest. 36, 469—473
22 Lernmark, A., Freedman, Z.R., Hoffman, C., Rubenstein, A.H., Steiner, D.F., Jackson, R.L., Winter, R.J. and Traismann, H.S. (1978) New Engl. J. Med. 299, 375—380