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Differences between individuals in high-molecular weight glycoproteins from mammary epithelia of several species

Stuart Patton [1], Gail E. Huston [1], Robert Jenness [2] and Yvonne Vaucher [3]

[1] *Center for Molecular Genetics, University of California, San Diego, La Jolla, CA,* [2] *Primate Research Institute, New Mexico State University, Holloman Air Force Base, NM, and* [3] *Department of Pediatrics, University of California, San Diego, La Jolla, CA (U.S.A.)*

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Milk fat globules are secreted by envelopment in plasma membrane of the lactating cell. SDS-gel electrophoresis of proteins from this membrane has revealed differences between human milk donors in two mucin-like glycoproteins. One of these glycoproteins resolves in 3% acrylamide stacking gel and the other in 4% running gel. The proteins vary in number of bands (one or two) and band mobilities. This polymorphism arises, at least in part, from expression of hypervariable genes. In this study, gel electrophoretic evidence of similar polymorphism in glycoproteins from cow, chimpanzee, horse and human milks is presented. In distinction to the other species, the cow expressed only one of these proteins which was detected in the running gel at M_r 180000 to 200000. The electrophoresis pattern for this protein from six cows was highly varied with respect to number (one or two) and position of bands. Peanut agglutinin, wheat germ agglutinin and concanavalin A all were bound specifically by bands of the bovine glycoprotein. Binding of concanavalin A distinguishes the bovine protein from the two human glycoproteins. Further studies of species differences should help shed light on the evolution of these unique glycoproteins and their possible functions in mother and young.

Introduction

Milk fat globules are secreted by envelopment in apical membrane (primarily plasmalemma) of the lactating epithelial cell [1–3]. Thus they display membrane glycoproteins on their surface. From the eight or more glycoproteins of the human globule membrane, Shimizu et al. [4,5] have partially characterized mucin-like components that are rich in serine and threonine and contain O-linked oligosaccharides. Initially a glycoprotein, PAS-0, of approximately 50% carbohydrate was isolated [4]. Subsequently another protein, called A, having 65 to 80% carbohydrate was obtained from the membrane. The A-protein could also be distinguished from PAS-0 by its lesser mobility during SDS-PAGE and by its binding of soy bean agglutinin and several monoclonal antibodies [5]. While the molecular weights of these human glycoproteins have not yet been determined, they are known as high-molecular weight glycoproteins (HM_r) because of their restricted mobilities in SDS-gels; i.e., much slower moving than reference proteins with molecular weights of 200000, such as myosin.

The HM_r glycoproteins have aroused considerable interest because of their usefulness in breast cancer screening. They are not expressed in the normal undifferentiated (non-lactating) mammary gland. However, some mammary carcinomas produce antigens that are detected by antibodies to the milk fat globule glycoproteins. These antibodies also offer a means by which therapeutic agents might be delivered to the carcinomas. Knowledge of the HM_r glycoproteins and their application in cancer detection have been reviewed [6]. There is evidence that they also occur in kidney tubules and urine [7,8].

The HM_r glycoproteins of the human milk fat globule exhibit differences between individuals [7,9,10]. The number, position, width and intensity of bands for these glycoproteins on SDS-gels varied among milk donors

Abbreviations: SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PAS, periodic acid Schiff's; PNA, peanut agglutinin; Con A, concanavalin A; FITC, fluorescein isothiocyanate; WGA, wheat germ agglutinin; PBS, buffer comprised of 0.137 M NaCl and 3 mM KCl in 10 mM phosphate (pH 7.2).

Correspondence: S. Patton, Center for Molecular Genetics, M-034J, University of California, San Diego, La Jolla, CA 92093, U.S.A.

and the pattern for an individual donor's glycoproteins was unchanged within and between lactations [9]. In the case of one (PAS-0), and probably both, HM_r glycoproteins, this individual-specific polymorphism results from hypervariability in the expressed genes [7,10]. These are the same type of genes as those used in DNA-identification of individuals [11].

We present evidence here that HM_r glycoproteins from milks of several other species (chimpanzee, horse and cow) also exhibit such polymorphism while showing species differences in the glycoproteins.

Materials and Methods

Human milk was collected from eight donors by electric breast pump (Medela Inc., Crystal Lake, IL) as described [12]. Six machine-milked cows, randomly selected out of a Holstein herd of 750, provided the bovine milk samples. Samples were obtained from seven unrelated chimpanzees by hand expression after anesthesia and administration of oxytocin. Seven mares, mostly thoroughbreds and including two half-sisters were milked by hand.

Milk fat globules were isolated [13] from fresh human and bovine milk samples. Some portions of bovine milk were made to 0.02% NaN_3 and stored at $-20°C$ before isolation of globules. These were used to establish reproducibility of gel patterns and to investigate the possibility of alteration of bands due to autolysis following milking. They were thawed and held at 37°C for 2.5 h before isolation of globules. All chimpanzee and equine samples were frozen at $-20°C$ immediately after milking and held at that temperature until analyzed.

Lipids were removed from suspensions of milk fat globules by a modification of the Roese-Gottlieb procedure [14]. The modification involved omission of NH_4OH and inclusion of a third extraction with 1.0 ml of diethyl ether per 0.2 ml of sample. Residual solvent was removed by N_2 aspiration and the sample restored to original volume with water. Protein content of extracted globule preparations was determined [15] using bovine serum albumin as standard.

SDS-PAGE of lipid-extracted globule and milk samples was conducted as described by Laemmli [16] and applied to human milk fat globule preparations [9]. Gels were stained for glycoproteins with Schiff's reagents and for proteins in general with Coomassie blue [17]. Reference proteins (Sigma, St. Louis, MO) were used to define relative molecular weight (M_r) positions of globule proteins on gels.

Lectin binding studies employed peanut agglutinin (PNA) and concanavalin A (Con A), both conjugated to FITC (E-Y Laboratories, San Mateo, CA), and wheat germ agglutinin (WGA) conjugated to Texas red (Molecular Probes, Eugene, OR). SDS-gels on which bovine globule proteins had been resolved were freed of stacking gel and fixed in 12.5% trichloroacetic acid at 2°C for one to several days. The fixed gels were washed with repeated changes of PBS containing 0.02% NaN_3 until pH 7.0 to 7.2 was achieved. They were then cut in half along the running axis to yield pieces $85 \times 68 \times 1.5$ mm. One half was placed in 25 ml of NaN_3-treated PBS containing 40 to 60 μg of fluorescent lectin per ml and the gel-lectin system was gently shaken for 48 h. Control experiments utilizing the other halves of gels, containing duplicate sample separations, were conducted to check the specificity of lectin binding. For this purpose, appropriate competitive inhibitors of binding were employed at 0.1 M concentration: D-galactose for PNA, *N*-acetyl-D-glucosamine for WGA and D-mannose for Con A. Lectin binding to gel bands was evaluated on a UV light box.

Results

We refer here to the HM_r glycoprotein of the human milk fat globule retained in a 3 to 4% stacking gel during SDS-PAGE as the A-protein, and that which penetrates a 4 to 6% running gel as the B-protein. The former corresponds to Component A of Shimizu et al. [4,5] and the latter to their Component B, C or PAS-0.

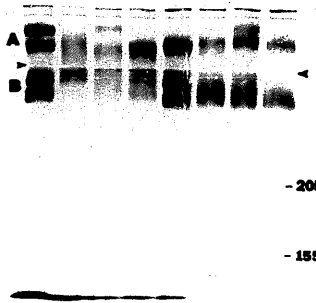

Fig. 1. An SDS-polyacrylamide gel showing electrophoretically separated glycoproteins of milk fat globules from eight human milk donors. Positions of the A- and B-glycoproteins are indicated at left. Note highly varied band patterns for both proteins. Samples each contained 100 μg of protein. The stacking and running gels were 3 and 4% acrylamide, respectively. Arrowheads indicate junction of these gels. Staining was with Schiff's reagents. Position of M_r references ($\times 10^{-3}$) are shown at right. Gel front (bottom) was partially electro-eluted.

These proteins are polymorphic [7,9,10]. A milk sample may exhibit one or two bands of either protein and the position of the bands on SDS-gels will vary from one individual's milk to another. Typical results for the HM_r glycoproteins of eight human milk donors are presented in Fig. 1.

By intensity of PAS-staining, the major glycoprotein of the bovine milk fat globule and its membrane is PAS-I [18–22]. It stains very weakly if at all with Coomassie blue [18–22]. On SDS-gels of 5 to 6% acrylamide, it exhibits a molecular weight of about 180000 to 200000 [6,18,23]. It is a protein by the criterion of proteinase degradation [21]. We have observed bovine PAS-I from samples of mixed herd milk as two close bands near the M_r 200000 reference marker on SDS gels [24,25]. It is shown in Fig. 2 that the number, position and staining density of bands for this protein vary among six individual cow globule samples (lanes 3–8) and that an equivalent mixture of the six milks yielded globules with the composite band pattern in lane 2. It is notable that other bovine glyco-

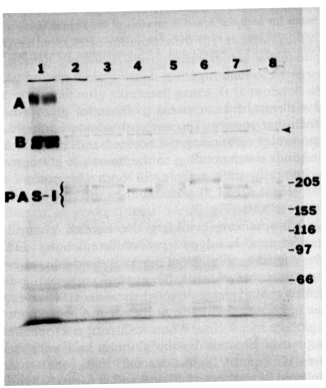

Fig. 2. An SDS-polyacrylamide gel showing electrophoretically separated glycoproteins from one human donor's milk fat globules, lane 1; from milk fat globules of six cows as a pooled sample, lane 2; and for the individual animals, lanes 3–8. Positions of the human A- and B-glycoproteins and bovine PAS-I are indicated at left. Note highly varied band patterns for PAS-I. Samples each contained 100 μg of protein. The stacking and running gels were 3.5 and 6% acrylamide, respectively. Arrowhead (right) indicates junction of these gels. Staining was with Schiff's reagents. Position of M_r references ($\times 10^{-3}$) are shown at right. Position of xanthine oxidase, the major bovine globule protein (1–3), which is Schiff's negative, is indicated at M_r 155000.

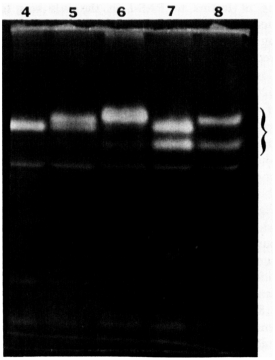

Fig. 3. An SDS-polyacrylamide gel revealing binding of the fluorescent-labelled lectin, PNA-FITC, to milk-fat-globule proteins of individual cows (lanes 4–8). Position of the PAS-I glycoprotein bands is indicated (bracket) at right of gel. Note that the varied patterns of PAS-I for the individual samples revealed by the lectin probe precisely match those shown for these samples by PAS-staining, Fig. 1, lanes 4–8. Samples and gel conditions were as in the legend to Fig. 1. For details of gel-lectin incubation, see text.

protein bands, lower on the gel, do not show such variability. This polymorphic protein is PAS-I based on the criteria that it is the strongest Schiff's-staining protein on the gel, that it does not stain with Coomassie blue, and that its M_r approximates the reported 200000. The much more intense staining of the human glycoproteins (lane 1, Fig. 2) in comparison to those of the bovine is impressive, as is the absence of any bovine bands in the stacking gel. PAS-staining for the bovine samples at the junction of the stacking and running gels (arrowhead) does not in our experience arise from an authentic glycoprotein band. Possible causes are anomalies in gel structure at the junction or incomplete solubilization of samples.

The band patterns of PAS-I from the six fat globule samples were precisely reproduced on additional gels. Globules prepared from the six milk samples that were frozen 3 weeks, thawed and incubated 2.5 h at 37°C produced the same gel band patterns as those from the freshly prepared globules.

All three of the lectins, PNA, WGA and Con A bound to gel bands of bovine PAS-I. Representative results with PNA are shown in Fig. 3. These data establish that the same varied set of bands revealed by PAS-staining are detected by FITC-PNA and this was true of the other two lectins as well. In all instances

binding of lectins to PAS-I in the gels was totally inhibited by the appropriate competing sugar. While the binding of PNA to bovine PAS-I seems not to have been reported previously, our positive results with WGA and Con A support the earlier observations of Murray et al. [26].

In Figs. 4 and 5, SDS-gels resolving glycoproteins in chimpanzee and mare milk samples are shown. The patterns of these glycoproteins are seen to vary among individuals as for the human and bovine. The A- and B-glycoproteins of the chimpanzees (lanes 2–8, Fig. 4) resemble bands for the human glycoproteins (lane 1, and Fig. 1), but the former tend to penetrate the gel somewhat more deeply. While the A- and B-glycoprotein bands of the horses (lanes 2–8, Fig. 5), appear fairly uniform in mobility, differences in band numbers are evident; e.g., some sample show one band of A-protein (lanes 2, 6 and 7) while others have two (lanes 3, 4, 5 and 8). Although not quite so clear in Fig. 5, such differences also occur in the B-protein. Bands for the horse glycoproteins (Fig. 5) are relatively fuzzy and streaked compared to those for human, chimp and cow. Repeated efforts to improve the band resolution, including analysis of lipid-extracted globule preparations, were unsuccessful. Bands for bovine PAS-I tend to be narrow

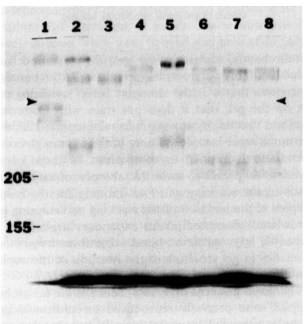

Fig. 4. An SDS-polyacrylamide gel showing electrophoretically separated glycoproteins from one human donor's fat globules, lane 1, and from samples of seven individual chimpanzees, lanes 2–8. Positions of the human A and B glycoproteins are indicated at left. Note the varied band patterns for both A and B proteins of the chimpanzees. Samples were a lipid-extracted suspension of human milk fat globules containing 100 μg of protein (lane 1) and 100-μl quantities of milk from which lipids had been extracted (lanes 2–8). The stacking and running gels, junction at arrowheads, were 3.5 and 5% acrylamide, respectively. Staining was with Schiffs reagents. Reference molecular weights ($\times 10^{-3}$) are at left.

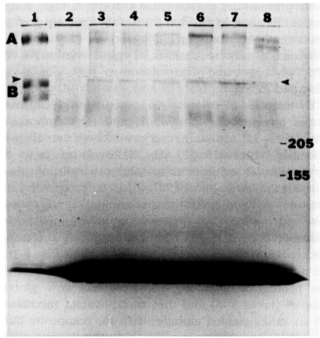

Fig. 5. An SDS-polyacrylamide gel showing electrophoretically separated glycoproteins from one human donor's fat globules, lane 1, and from milk samples of seven individual mares, lanes 2–8. Positions of the human A and B proteins are indicated at left. Note variations in the bands for both the A and B proteins of the horses. Samples and gel conditions were as in caption, Fig. 3, except that 100-μl quantities of extracted mare's samples and a 6% acrylamide running gel were used.

and well resolved compared to those for glycoproteins of the other species. This suggests greater homogeneity in molecules comprising the bovine bands. It is notable that bands corresponding to the human A glycoprotein are found in milk samples of both chimpanzee and horse (Figs. 4 and 5) whereas they are missing in the bovine samples (Fig. 2).

In comparisons involving the human, chimp and horse species, band patterns of fat globules and of corresponding milk for a given individual, were the same (data not shown). This seems reasonable since Shimizu et al. [5] have detected the same HM_r glycoproteins in human skim milk as they found in human milk fat globule membrane. When we attempted to make the comparison between bovine globules and milk, bands from 100 μl of lipid-extracted milk, the quantity employed in analyses of Figs. 3 and 4, were too faint for evaluation. This suggests either much less glycosylation and/or much lower concentration of the glycoprotein in bovine milk as compared to milk of the other species.

Discussion

The bovine milk fat globule glycoprotein, PAS-I, appears to be related to the HM_r A- and B-glycoproteins of the human milk fat globule. PAS-I has the same type of banding diversity between individual on SDS-

gels as do the human glycoproteins, i.e., one or two bands of varying mobility (compare Figs. 1 and 2). The glycoproteins of both species contain relatively high levels of serine and threonine [4,5,27]. A further resemblance is the affinity of bovine PAS-I for PNA and WGA in the manner of the human glycoproteins [4,5]. It seems likely that milks of most species contain PAS-I-related proteins. The human B-protein is reported to bind several monoclonal antibodies raised against PAS-I of guinea pig milk fat globules [28,29]. A similar type of polymorphism is evident in the A- and B-glycoproteins of chimpanzee and mare milk (Figs. 4 and 5). Why there is only one HM_r glycoprotein in the cow globule remains to be answered; and until structures of the bovine and human glycoproteins are examined more thoroughly, it will not be certain whether bovine PAS-I is more closely related to human A- or B-glycoprotein. The observation (Ref. 26 and herein) that bovine PAS-I binds Con A, whereas the human A- and B-glycoproteins do not [4,5], is one indication of significant structural difference between the proteins of the two species. The spectrum of milks available from primitive to more modern mammals affords an opportunity to investigate the evolution of these proteins and to obtain evidence of their function(s).

Gendler et al. [10] have shown that polymorphism of the human globule B-glycoprotein is due substantially to the protein moiety which is the product of hypervariable co-dominantly expressed genes. It is probable that the A-protein, too, is the product of such genes since it exhibits the same kinds of variations between individuals on SDS-gels as does the B-protein (Fig. 1). Polymorphism in the oligosaccharides is also possible in view of blood group activity of both oligosaccharides [30] and glycoproteins [31] of human milk. In addition, the movement of glycoproteins during SDS-PAGE can be significantly influenced by their degree of sialylation [29]. The general availability of bovine milk and tissues, as well as milk samples from individuals of large bovine families, including lactating identical twins and triplets, should facilitate molecular genetic investigation of PAS-I polymorphism. A comparison of 10 Holstein dams with 12 of their daughters for mobility of PAS-I bands on SDS-gels revealed that in all cases one of the daughter's bands matched one of her mother's (Patton, S., Huston, G.E. and Patton, R.S., unpublished data). Usually the daughters' second band, when observable, were unmatched. This implies that co-dominant genes code for the two protein bands, one inherited from the sire and the other from the dam, as shown for the human B-glycoprotein [7,10].

An approximate carbohydrate content of 50% has been estimated for both bovine PAS-I [27] and the human B-protein [4]. This infers that the much more intense Schiff's staining of the human compared to the bovine protein (Fig. 2) is due to a lower concentration of the latter in the total globule protein. Reduced protein glycosylation by the bovine mammary gland may also be involved in this difference. There is approximately one-tenth as much free oligosaccharide in bovine milk as in human milk [32]. This apparent difference in glycoprotein concentration suggests greater benefit from these complex forms of carbohydrate in milk for the young human than for the calf. One manifestation of difference in globule glycoproteins between the two species is that they constitute filament-like structures which can be seen by freeze-etch electron microscopy on globules from human and horse but not on bovine globules [24,25]. Based on their individual-specific nature and cell surface location on the mammary cell, it has been suggested [9] that the HM_r glycoproteins may also have a role in immuno-recognition phenomena.

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