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Sequential Changes in the Antimicrobial Protein Concentrations in Human Milk during Lactation and Its Relevance to Banked Human Milk

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ABSTRACT. The concentrations of eight antimicrobial proteins, 11S IgA, IgG, IgM, α_1-antitrypsin, lactoferrin, lysozyme, C3, and C4 were measured in sequential samples of colostrum, transitional and mature milk from 47 women in Merseyside, England by single radial immunodiffusion. Concentrations were highest in colostrum and then declined until relatively stable concentrations were reached in mature milk. A wide variation in protein concentrations was noted in the milk from different individuals at any given postpartum time interval and this was particularly so during the first 4 days postpartum. The decline in individual antimicrobial protein concentrations seen in samples of transitional milk appeared to take a variable period of time to stabilize to mature milk concentrations of each protein. These variations may reflect different rates of transport or secretion of these proteins from the alveolar epithelial cells into the alveolar lumen. Because of the wide variations in antimicrobial proteins observed in milk samples from the individuals in this study at similar postpartum time intervals, it is suggested that banked milk should be monitored not only for bacterial contamination, but also for levels of antimicrobial proteins, if it is considered that a major advantage of unprocessed human milk is the immune protection which it confers. (*Pediatr Res* 19:561–565, 1985)

Since 1975 several workers have measured the concentration of certain protective proteins in human colostrum and milk which are available to the newborn for passive immunity (1–7). Others have measured not only the concentration, but also the total daily transfer of these proteins to normal breast-fed babies (8, 9). Haneberg and Aarskog (10) found that there were rapid changes in volume and composition of the milk during the first week after delivery, which corresponded with the time when the infant's own defence mechanisms were least developed, particularly the mucosal production of secretory IgA. This vital period of development, without a fully functioning immune system, is seemingly dependent upon passively transferred immunity to provide local protection in the neonatal intestine and possibly to play some part in protection against such processes as the transfer of macromolecules and/or organisms into the systemic circulation from the gut.

All previous workers have noted that protective protein concentrations in milk samples fall rapidly during the first 7 days postpartum but, whereas the pattern of protein decline is similar in all cases for each individual protein, there is a marked individual variation in concentrations. This variation in antimicrobial protein content is important when considering pooled banked human milk. It would seem advisable that only milk of reasonable protective potential should be given to low birth weight and other "at risk" neonates if it is accepted that the major advantage of unprocessed human milk is the immune protection which it is thought to confer.

This study was performed to investigate individual variations in protective milk protein concentrations during the entire period of lactation in 47 women from Merseyside, England.

MATERIALS AND METHODS

Forty-seven mothers of healthy babies who were delivered "normally" at or around full-term, donated milk samples by manual expression at the end of the second feed of the day on sequential days following delivery. Any sample donated during the first 24 h was designated day 1 and thereafter every 24 h as days 2, 3, 4, etc. Ideally a sample was required every day for the first 7 days and then once or twice weekly if possible until the cessation of breast-feeding. Samples were expressed into sterile universal containers and immediately placed in the freezing compartment of the ward or home refrigerator and later transferred to the laboratory and stored at −20° C. The number of samples donated by the volunteers during each time interval is shown in Table 1.

Laboratory methods. The concentrations of IgA (secretory 11S IgA), IgG, IgM, α_1-antitrypsin, lactoferrin, lysozyme, B_1A globulin (C3) and B_1E globulin (C4), were determined in all samples by single radial immunodiffusion (11) in 1% agarose, each sample being tested in duplicate on each of two separate immunodiffusion plates at two or three different dilutions. Samples were defatted by centrifugation at 25,000 × *g* at 4° C for 30 min prior to testing. Goat antisera to IgA, IgG, IgM, and lactoferrin (Hyland Pharmaceuticals and Nordic Immunologic Laboratories) were used. For α_1-antitrypsin, B_1A globulin (C3) and B_1E globulin (C4), rabbit antisera (Hoechst Pharmaceuticals) were used. Standardized human colostrum (kindly provided by D. B. McClelland, J. M. McGrath, and R. R. Samson) was used as standards for 11S secretory IgA, lactoferrin, and lysozyme. Tripartigen prediluted standards (Hoechst Pharmaceuticals) were used for IgG and IgM. A protein standard plasma was used for α_1-antitrypsin and standard human serum was used for B_1A globulin (C3) and B_1E globulin (C4).

RESULTS

The median concentrations of the eight antimicrobial proteins in breast milk obtained during each time interval post partum

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Table 1. *The median concentration, the range and the interquartile range (IR) of each antimicrobial protein from all milk samples obtained from the 47 donors during each time interval*

Days postpartum		1	2	3	4	5	6	7
	*n**	12	30	40	30	31	25	16
IgA	Median	6270	6924	2086	755†	489	396	360
	IR	2100–11520	2000–11360	1080–4320	450–1160	356–650	325–560	170–490
	Range	440–21840	620–25760	230–25760	130–11200	190–5920	150–7040	125–715
IgG	Median	52.7	41.7	13.7	6.0†‡	5.7	5.4	4.6
	IR	23.0–112.0	25.0–116.0	10.4–26.4	4.2–8.9	3.6–7.0	3.3–6.6	3.8–5.7
	Range	4.4–676.0	2.6–644.0	2.6–235.0	2.4–30.5	1.8–13.2	2.1–12.2	3.3–10.6
IgM	Median	60.0	70.0	28.8	7.8†	4.7	6.6	4.7
	IR	24.0–186.0	42.4–111.0	11.6–49.6	5.3–17.4	3.7–8.8	3.1–9.1	3.4–7.2
	Range	2.6–344.0	21.4–744.0	4.8–400.0	2.0–76.4	2.0–136.0	2.0–20.0	2.7–21.2
α_1-Antitrypsin	Median	71.5	54.3	25.2	17.7†	16.4	18.0	16.6
	IR	37.5–124.5	40.00–92.5	17.8–39.2	15.2–22.6	11.2–20.0	13.2–20.8	10.8–18.0
	Range	16.0–200.0	17.6–230.0	10.8–150.0	3.0–45.6	4.4–37.8	4.7–31.6	4.9–31.6
Lactoferrin	Median	1650	1794	1100	530†	640	720	400
	IR	1360–3200	1120–2320	595–1960	300–1500	320–1266	300–1020	240–830
	Range	125–4020	500–8240	120–6320	80–3640	120–2600	120–3300	80–4440
Lysozyme	Median	38.3	34.2	15.5	10.6†	9.4	10.2‡	8.6
	IR	24.4–69.5	22.5–50.0	10.0–31.0	8.2–18.6	8.0–12.8	5.8–15.8	6.8–11.4
	Range	16.0–226.0	7.3–250.0	3.8–100.0	5.4–57.5	4.6–95.0	3.8–62.0	4.6–55.0
C_3	Median	67.0	40.2	14.0	6.1†	3.8	3.1	3.4
	IR	42.0–90.0	32.5–64.5	9.5–30.5	4.8–8.4	2.8–4.2	2.2–4.8	2.5–4.0
	Range	28.0–142.0	8.5–244.0	7.5–118.0	2.2–25.0	1.1–48.3	1.3–9.0	1.2–9.6
C_4	Median	26.5	16.1	6.1	3.6†	3.4	3.8	4.0
	IR	24.8–35.0	11.0–24.8	3.9–9.6	2.3–4.8	2.1–5.7	2.0–6.0	2.8–5.5
	Range	9.0–68.0	6.2–200.0	1.7–54.0	1.5–23.0	1.5–15.6	1.7–16.5	1.7–16.4

Days postpartum		8–14	15–21	22–28	29–42	43–56	57–70	71–84
	*n**	34	36	37	12	30	40	30
IgA	Median	253‡	235	205	193	189	183	181
	IR	195–365	196–360	170–260	165–265	155–230	145–285	135–213
	Range	115–754	90–685	115–665	105–535	110–665	105–540	105–470
IgG	Median	5.1	4.6	4.6	3.7	4.4	5.0	4.2
	IR	3.6–6.7	3.9–6.3	3.4–5.7	3.2–5.4	3.5–5.7	3.4–6.3	3.5–5.2
	Range	2.9–12.5	2.6–12.0	2.4–18.1	1.9–12.4	2.3–12.7	1.9–15.0	2.0–8.3
IgM	Median	3.6	2.3	2.0	2.5‡	2.0	1.9	1.8
	IR	2.5–5.0	1.7–3.9	1.8–3.1	1.6–3.0	1.3–2.3	1.3–2.6	1.2–2.2
	Range	1.5–15.9	0.0–11.7	1.2–8.6	0.9–6.7	0.8–4.6	0.0–17.1	0.0–3.5
α_1-Antitrypsin	Median	9.1	6.5	6.2‡	4.7	4.8	5.0	4.0
	IR	7.3–13.6	5.0–9.7	5.0–7.4	4.0–6.4	3.5–5.7	4.0–6.2	3.5–4.8
	Range	3.2–23.8	1.1–18.6	1.1–11.9	1.0–14.7	1.8–8.5	1.9–54.8	1.9–6.2
Lactoferrin	Median	420	405	385	295	250	266‡	105
	IR	288–570	255–565	165–530	150–390	115–385	120–440	25–285
	Range	120–2040	36–1430	20–935	5–705	5–685	13.5–675	6.5–563
Lysozyme	Median	7.4	5.4	6.8	6.6	12.3	10.6	12.8
	IR	5.0–13.1	4.3–9.6	4.2–12.0	3.6–12.4	5.6–18.8	4.8–24.8	7.0–19.0
	Range	3.2–58.5	1.2–62.3	2.3–75.5	1.0–99.5	2.2–84.0	3.5–72.0	2.4–54.3
C_3	Median	1.9	1.5‡	1.1	1.0	1.1	1.0	0.9
	IR	1.3–2.9	1.1–2.1	0.9–1.5	0.8–1.5	0.8–1.6	0.8–1.0	0.8–1.0
	Range	0.9–8.2	0.6–6.2	0.6–2.9	0.6–2.7	0.4–3.4	0.5–26.8	0.5–2.1
C_4	Median	2.7	2.5‡	1.8	1.8	1.8	1.8	1.6
	IR	2.0–4.3	1.8–3.6	1.6–2.4	1.5–2.7	1.6–2.4	1.6–2.7	1.3–1.9
	Range	1.4–12.6	1.2–7.0	1.1–5.3	1.4–13.4	1.2–9.7	1.3–8.6	0.7–3.2

Days postpartum		85–98	99–112	113–126	127–140	141–154	155–168
	*n**	31	25	16	34	36	37
IgA	Median	185	190	160	183	170	175
	IR	145–215	135–205	135–190	110–230	135–185	135–145
	Range	100–350	100–340	115–480	100–400	105–590	128–620
IgG	Median	3.9	5.1	4.0	4.0	5.0	5.2
	IR	3.1–5.6	3.2–6.1	3.3–4.8	3.5–5.3	3.4–6.3	3.1–7.0
	Range	2.2–10.2	1.6–10.1	2.5–11.2	3.0–8.1	2.9–9.3	2.9–11.4
IgM	Median	1.6	1.6	1.6	2.0	1.8	1.8
	IR	1.4–2.0	1.0–2.0	1.3–2.0	1.1–2.1	1.4–2.1	1.2–2.2
	Range	0.0–3.3	0.0–3.0	0.0–3.5	3.0–8.1	0.9–10.3	0.9–2.6

Table—*continued*

Days postpartum		1	2	3	4	5	6	7
	*n**	12	30	40	30	31	25	16
α_1-Antitrypsin	Median	4.3	4.6	4.5	4.5	4.4	5.4	
	IR	3.3–5.1	2.7–6.0	3.3–5.2	3.8–5.9	3.8–5.9	3.7–5.9	
	Range	1.9–9.0	1.2–9.1	1.6–7.3	2.6–8.1	3.8–8.8	3.5–7.4	
Lactoferrin	Median	250	245	115	130	135	165	
	IR	58–318	80–370	62–178	30–370	55–483	30–395	
	Range	4–625	3–750	5–588	5–440	25–1370	25–695	
Lysozyme	Median	15.0	13.4	11.7	20.2	21.0	19.8	
	IR	10.8–24.4	7.2–34.0	5.4–20.7	11.0–26.5	11.8–28.5	7.9–49.0	
	Range	2.2–91.7	2.2–189.0	2.5–112.3	6.9–75.0	2.0–81.0	7.1–123.0	
C_3	Median	1.0	1.0	0.8	0.9	0.9	0.8	
	IR	0.8–1.3	0.8–1.4	0.7–1.0	0.7–1.3	0.7–1.1	0.7–1.3	
	Range	0.7–2.2	0.5–2.1	0.6–4.2	0.4–1.8	0.6–2.9	0.7–4.4	
C_4	Median	1.6	2.0	1.5	1.9	1.5	1.6	
	IR	1.3–2.3	1.3–2.3	1.3–2.4	1.4–3.0	1.4–2.7	1.3–2.6	
	Range	0.0–3.9	0.0–3.9	0.0–5.5	1.2–4.9	1.2–11.0	1.0–7.3	

* Number of samples donated during each time interval.

† Indicates on which day the initial rapid drop in protein concentrations occurs.

‡ Indicates on which day the gradual drop in protein concentrations ceases and subsequent samples have relatively constant concentrations (except for lysozyme).

from all 47 volunteers is shown in Table 1. The range, interquartile range, and number of samples obtained during each time interval is also shown.

Among the eight proteins measured there were several distinct patterns of variations of the protein concentrations during lactation.

IgA. Median concentrations of IgA fell very rapidly over the first 4 days from a concentration approximately 30 times the normal serum level (6270 mg/dl) to levels three to four times that found in serum (755 mg/dl). Concentrations then showed a gradual decline from day 4 to 7 to concentrations of 360 mg/dl. Over the next 6 months, concentrations, although declining very slightly, remained relatively constant, never falling below serum IgA levels.

IgG. Median IgG concentrations fell very rapidly over the first 4 days from only 5% of the normal serum levels (52.7 mg/dl) to very low levels by day 4 (6.0 mg/dl). Thereafter concentrations remained relatively constant.

IgM. Initially the IgM median concentration was approximately half the normal serum level (60.0 mg/dl) and over the next 24 h rose slightly (70.0 mg/dl). Concentrations then fell rapidly to 5% of serum levels by day 4 (7.8 mg/dl), then more gradually from day 4 to 29–42 (2.5 mg/dl) and thereafter remained relatively constant.

α_1-Antitrypsin. The median concentrations fell rapidly over the first 4 days from a value approximately 35% (71.5 mg/dl) to approximately 8.5% (17.7 mg/dl) of the normal serum levels by day 4. Concentrations then fell more gradually from day 4 to 22–28 (6.2 mg/dl) and thereafter remained fairly constant.

Lactoferrin. Lactoferrin median concentrations fell rapidly from day 1 (1650 mg/dl) to day 4 (530 mg/dl), then more gradually until days 57–70 (266 mg/dl) and thereafter remained fairly constant.

Lysozyme. The median concentrations of lysozyme fell rapidly from day 1 (38.3 mg/dl) to day 4 (10.6 mg/100dl) and then more gradually until day 7 (8.6 mg/dl). After this they remained fairly constant until days 29–42 and thereafter showed a gradual rise.

C3. C3 median concentrations fell rapidly over the first 4 days from a value approximately one and a half times the normal serum level (67.0 mg/dl) to approximately 15% (6.1 mg/dl) of normal serum levels. Concentrations then fell more gradually from day 4 to 15–21 (1.5 mg/dl) and thereafter remained fairly constant.

C4. Median C4 concentrations fell rapidly from levels twice those in serum on day 1 (26.5 mg/dl) to levels approximately 30% of normal serum values by day 4 (3.6 mg/dl). Concentrations then fell more gradually until days 15–21 (2.5 mg/dl) and thereafter remained fairly constant.

Among the 47 donors it was seen that there was a very wide range of protein concentrations in the milk donated by different individuals at each time interval considered. This is further supported by the ranges and interquartile ranges of concentration for each antimicrobial protein on each of the days postpartum as seen in Table 1. The individual variation in IgA protein concentrations from the 47 individuals on each of the first 4 days postpartum is shown on Figure 1. At a given time, protein concentrations in individual milk samples are, to a reasonable approximation "lognormally" distributed (ie, logarithms of concentrations have an approximate normal distribution), apart from the occasional zero reading. On this assumption, the numbers of samples with extreme values (ie, lowest 5% and highest 5% observed) are more or less as expected from normal 90% tolerance limits.

DISCUSSION

Of the eight antimicrobial proteins studied, the three produced locally in the breast, secretory IgA (plasma cells), lysozyme, and lactoferrin (secretory epithelial cells), are found in very high concentrations in colostrum and milk. Secretory IgA and lysozyme levels are higher than serum levels even in mature milk while lactoferrin is found only in trace amounts in normal serum (12). The other proteins studied, which are actively transported into the milk from the maternal serum, are found in much lower concentrations although similar trends in concentration declining with time are seen in colostrum and milk. By the end of the first month of lactation, many of the host defense factors that are present during the first days of lactation, fall to very low levels and the majority of the protein then consists of α-lactalbumin, casein, and other proteins which presumably have a primarily nutritional function. Secretory IgA, lysozyme, and lactoferrin, however, continue to be transferred to the infant in considerable quantities since they still represent a substantial proportion of the total milk protein (8). This emphasises that breast milk may be particularly important during early neonatal life in defense against infection, in handling ingested macromolecules and in maintaining a natural intestinal microbial flora.

Although not considered in this study, it must be emphasized that there are many other soluble antigens in human milk which have been shown to inhibit certain microorganisms by combin-

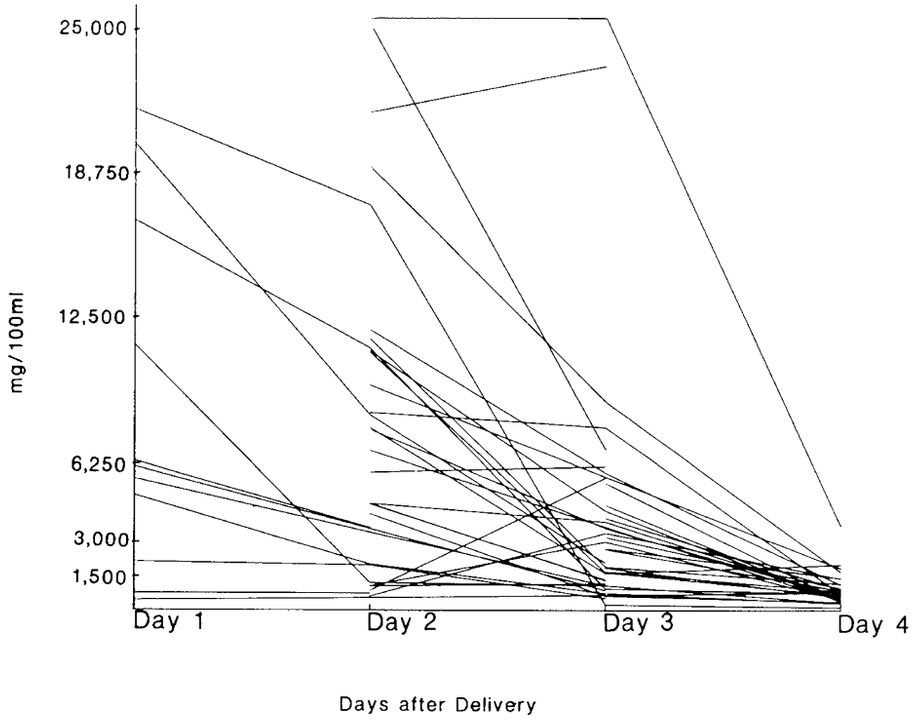

Fig. 1. The individual variation in IgA protein concentrations from the 47 volunteers on each of the first 4 days postpartum. (Missing values are due to an individual being unable to produce a sample of milk on that particular day.)

ing or competing with them *in vitro*; these include secretory piece, lactoperoxidase, interferon, bifidus factor, and immunoregulatory mediators (13). Many of these factors may function in conjunction with the specific immunoglobulins and components of the complement system. Other immunologically reactive factors have been identified but the role of these proteins in colostrum and milk-mediated protection in the newborn infant remains to be determined (14).

Colostrum is usually defined as the milk produced during the first 48–72 h following parturition and in this study it has been shown that antimicrobial protein concentrations fall very rapidly beyond this time and continue to do so for a further 24 h in all cases. It would, therefore, seem logical that milk produced within the first 96 h postpartum should be considered as colostrum. The then more gradual drop in concentration until relatively constant mature milk protein concentrations are reached is the milk termed "transitional milk." During this time there is a higher antimicrobial protein concentration than in mature milk but over a few days this concentration gradually decreases while the milk volume gradually increases (15). In this study the decline in individual antimicrobial protein concentrations seen in samples of transitional milk appears to take a variable period of time to stabilize to the mature milk concentrations of each protein. IgA and lysozyme took 3 days, IgM and α_1-antitrypsin took between 18 and 24 days, C3 and C4 took between 11 and 17 days, and lactoferrin between 25 and 38 days. IgG concentrations had reached approximate mature milk levels by day 4. These variations may reflect different rates of transport or secretion of these proteins from the alveolar epithelial cells into the alveolar lumen.

Before parturition much antimicrobial protein has been stored in the ductular system of the breast and its secretion postpartum is probably the cause of the highly concentrated colostral proteins secreted during the first 4 days of lactation and it is probably not included in transitional milk. Following parturition and expulsion of colostrum from the ducts of the breasts in response to early suckling, accumulated secretory product within the alveolar epithelial cells is extruded and enters the alveolar lumen (16). Protein present is diluted by lactose release and its osmotic drag on water, and when this is secreted as milk with any remaining colostrum from the ducts of the breast, its concentration will be less than colostrum but more than mature milk. Following this process the lumen of the alveolus dilates due to the increased volume of milk and the alveolar epithelial cells become flatter with little secretory product within their cell cytoplasm (16). Active transport of protein from either the extracellular space or the epithelial cell itself is then required to move antimicrobial proteins into the alveolar lumen. This transport may have a relatively constant basal rate and this would explain the subsequently "stable" concentrations of these proteins in the mature milk of each individual. Variations in the concentrations of the antimicrobial proteins in the mature milk of different individuals, although small, probably reflect different volumes of secretory alveoli in the breast and different basal rates of protein synthesis in the breast lobules or different rates of transport of protein from the maternal circulation into milk. It is interesting to note that there is no evidence of increased antimicrobial protein concentrations in the breast milk of hypersecretors of precolostrum during their postpartum "transition time" so that following the 4th day postpartum, antimicrobial protein concentrations have reached the mature milk levels (17). This probably reflects the fact that these individuals have increased lactose secretion from alveolar epithelial cells during later pregnancy and that this facilitates the more rapid clearing or "flushing out" of accumulated protein from the mammary alveolar and ductular system. Extrusion of accumulated secretory product within the alveolar epithelial cell cytoplasm may also be facilitated.

The present study clearly demonstrates a wide variability in the concentrations of eight antimicrobial proteins present in colostrum and transitional milk, but there is a much smaller variability in their concentrations in mature milk although the concentrations are much lower. Where mature milk is used to supply hospital milk banks, it is, therefore, likely to contain lower concentrations of immunologically protective proteins than banked milk containing colostrum or transitional milk. This may be relevant to the feeding of preterm and sick neonates

where the protective capabilities of human milk may outweigh its nutritional benefits. Satisfactory nutritional intake for these infants may in fact be better supplied using artificial feeds at present (18). It is, however, recognized that there is an immunochemical difference between the milk of women delivering prematurely (preterm milk) and those delivering at term (19). IgA concentrations are reported to be significantly higher in preterm milk (4, 19) and although there is little information with regard to the concentrations of other antimicrobial proteins and their physiological and immunological effects, the preterm infant would appear to receive milk from its mother with a higher protective potential than term or mature milk. It seems logical, therefore, in cases where immune protection is most needed, to use colostral or transitional milk feeds where protective protein concentrations are highest and most closely resemble those found in preterm milk.

Among the 47 women studied there were several individuals with very low levels of the eight protective proteins measured in their colostrum and early milk; eg, one idividual had IgA levels as low as 440 mg/dl on day 1 postpartum. The other antimicrobial proteins measured in the milk of this individual were similarly of low concentration. Theoretically such milk might confer less protection against infection and other immunological stimuli although to date this has not been demonstrated *in vivo*. Logically, however, it is suggested that all milk from mothers breastfeeding low birth weight or at risk babies, and all milk donated to milk banks might be screened not only bacteriologically, but also for the concentration of some of its major immunologically protective proteins, to ensure adequate immunological potential.

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