Lactoperoxidase Activity in Human Milk and in Saliva of Newborn Infants

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Human milk and saliva from newborn infants were analyzed for their content of lactoperoxidase and thiocyanate. The activity of lactoperoxidase in infant saliva was variable but generally higher than that found in calf saliva. In contrast, the activity in human colostrum was low (~5%) compared with that found in cow's milk. The enzyme was resistant to gastric juice. Thiocyanate was demonstrated in infant saliva in concentrations about one-third of that in adult saliva. The amounts of lactoperoxidase and thiocyanate in infant saliva are quite sufficient to inhibit bacterial growth in in vitro systems. The importance of this system in vivo has not yet been demonstrated. The availability of this system to both newborn calves and humans (in calves provided largely by colostrum and in human babies by saliva) might be indirect evidence of its importance.

Saliva, tears, and the milk from many different mammals have peroxidase activity. In cows, immunological and chemical investigations (23) have shown that the activity is due to the same enzyme in all three fluids: lactoperoxidase. The enzyme appears, together with thiocyanate in physiological concentrations and H₂O₂, to have an antimicrobial function, which has been intensively studied in vitro (3, 9, 11, 12, 17, 25, 29, 30). The effect in vivo is, however, uncertain (19; H. Hoogendorn, Ph.D. thesis, Technische Hogeschool Delft, The Netherlands, 1974). In human newborn infants, bacterial colonization starts immediately after birth. The factors governing this colonization and preventing clinical infections are not very well known. Human milk is rich in factors that may protect the recipient infant against infections (10). Since the quantities of most of these factors are greater in human than in bovine milk, it has been proposed that breast feeding, on this basis alone, is to be preferred (21). The lactoperoxidase system is said to be one of these factors (10). A full evaluation of the availability of this system to newborn infants has not been made. One study showed only low concentrations of lactoperoxidase in human milk (15), and no investigations regarding the content of the enzyme in saliva of newborn infants have been performed. The presence of thiocyanate apparently has not been studied in saliva from newborn infants. Hydrogen peroxide can be produced by bacteria (11) and, conceivably, also by certain oxidases in milk and from auto-oxidation of various substances. The present study is part of an examination of different factors influencing the colonization of newborn infants. Our aim is to explore the availability to the infants of the different components of the lactoperoxidase antibacterial system. Supplementary analyses of lactoperoxidase in cow's milk and in calf saliva are also presented.

MATERIALS AND METHODS

Breast milk. Ninety-five specimens were obtained from 45 mothers during 6 weeks before to 9 weeks after delivery. The samples were obtained either by manual expression or by a breast pump just before the infants started a meal; i.e., 4 and sometimes 8 h had passed since the breast last was emptied. The specimens were kept at 4 C for no more than 4 h. After centrifugation (10,000 x g for 10 min), the fatty layer was removed and discarded and, leaving the bottom pellet, the milk was collected and stored at ~70 C.

Saliva. A total of 139 specimens was obtained from 75 healthy infants. From 20 infants, three or more samples were collected during a period of 9 weeks after birth. Each specimen was collected at 10 a.m. or 2 p.m., just before nursing. The saliva was collected with a polyvinyl catheter (feeding tube, French size 5, C. R. Bard International Ltd., England) adapted to a plastic syringe. The saliva was transferred to glass tubes, sealed with Parafilm (American Can Co., Neenah, Wis.), stored at 4 C, and analyzed within 3 days. Controls showed that the peroxidase activity was unchanged during more than 2 weeks at 4 C. Twenty-six healthy adults working in the laboratory served as a control group. The unstimulated saliva was delivered in glass tubes and then treated like infant saliva.

Bovine specimens. Colostrum (milk) was collected by hand milking once during the first 24 h after
parturition and also on days 3, 5, and 14. On the same day, saliva was obtained from three newborn calves. The specimens were treated like the human specimens.

**Peroxidase assay.** Saliva or milk serum was added to 16.7 mM pyrogallol in 0.2 M sodium phosphate buffer, pH 6.0 (total volume, 3.0 ml). Hydrogen peroxide, final concentration 1.67 mM, was added and the peroxidase activity was calculated from the initial rate of increase in absorbance at 400 nm. The activity was expressed as \( \Delta A_{400} \) per minute per milliliter of milk or saliva. The use of a high concentration of pyrogallol, which has a very high reactivity with the "peroxide compounds" of lactoperoxidase (6), makes the method relatively insensitive to interference by compounds acting as peroxidase substrates.

The standard deviation for a series of determinations is \( \pm 5\% \). The reproducibility of the method for a certain amount of human saliva (kept at -70°C) over a long time (months) is better than \( \pm 10\% \). The maximal difference between the results of adding very little or very much of one specimen of human saliva to the assay medium is less than 20%. In general, the points in the figures represent one or two determinations.

**Possible contribution by leukocytes to the peroxidase activity.** Human milk contains about 2,000 leukocytes per \( \mu l \) (10). Human saliva also contains leukocytes, but the content before dental eruption is very low (28). Several types of leukocytes—polymorphonuclear granulocytes, eosinophil granulocytes, and monocytes (1, 2, 26)—contain peroxidases and may hence contribute to the peroxidase activity of milk and saliva. Therefore it was ascertained that the contribution from leukocytes was negligible and that the activity found was due to lactoperoxidase: the milk (but not saliva) was always centrifuged and the sediment containing leukocytes was discarded. Furthermore, a suspension of human leukocytes from blood was prepared (5) (2,000 cells/\( \mu l > 80\% \) polymorphonuclear granulocytes) and its peroxidase activity was investigated. The peroxidase assay (see above) is performed in a slightly hypotonic medium, and the suspension was found to exhibit no peroxidase activity. After homogenization of the suspension in hypotonic medium, a low activity was found (\( \Delta A_{400} = 0.18/\text{min per ml of suspension} \)). Thus, even if leukocytes in milk and saliva are lysed, their contribution to the observed peroxidase activities is insignificant.

**Variation with time of peroxidase activity in saliva.** From six infants, 2 or 3 days old, consecutive samples of saliva were obtained during 1 day. The specimens were collected just before nursing, which started at about 6 a.m., 10 a.m., 2 p.m., 6 p.m., and 10 p.m., and treated as previously described.

**Effect of gastric juices on peroxidase activity.** (i) Gastric juice was obtained from one adult (pH 1.2) and from one infant with pyloric stenosis (pH 2.3). Milk samples were obtained from one cow the day after parturition and from 15 mothers a few days before delivery (precolostrum) or a few days after delivery (colostrum). The maternal milk was divided in three parts: (i) pool of 10 samples of precolostrum; (ii) pool of two samples of colostrum; and (iii) one sample of colostrum. Gastric juice and milk (1:5) were incubated at 37°C. This ratio between gastric juice and milk is higher than can be expected during a normal meal (20, 22). The final pH of the gastric juice-milk mixtures was about 5. At that pH the peptic activity should be very low. Aliquots were removed after 0, 15, 30, 60, 120, and 240 min, brought to neutral pH to stop all peptic activity, and stored at -70°C. As a control, a mixture of saline and milk (1:5) was incubated for 240 min.

(ii) Colostrum (100 ml) was given to the infant with pyloric stenosis and aliquots of the stomach content were obtained after 0, 15, and 30 min, after which the infant underwent pyloromyotomy. After neutralization, the aliquots were stored at -70°C. The peroxidase activity was assayed as described above.

**Thiocyanate assay.** The thiocyanate content of milk and saliva was determined as ferrithiocyanate after precipitation of protein with trichloracetic acid (8).

**RESULTS**

**Breast milk.** The lactoperoxidase activity in precolostrum, colostrum, and mature human milk is shown in Fig. 1. The median value of the enzyme activity declined from 3.28 in precolostrum to 0.51 in 1 week and 0.34 in 9 weeks after delivery. The range changed from 0.3 to 11.7 in precolostrum to 0 to 0.49 in 9 weeks after delivery.

**Infant saliva.** The enzyme activity in the infants' saliva during the first few days after birth showed marked variation in consecutive specimens (Table 1) from the same individual. An infant who showed no activity at all could a few hours later have a high activity or vice versa. Thus, lactoperoxidase activity is not continuously demonstrable in the saliva of newborn infants. On the other hand, extremely high values were also observed (Table 1, and Fig. 2). These extremes were never seen in adult saliva, where activity always was demonstrable and within narrow limits compared with the newborn infants.

**Inhibitors of the lactoperoxidase assay.** To test whether or not inhibitors were present in saliva, peroxidase-containing saliva was added as internal standard to several of the salivas devoid of activity. No inhibition was observed. The experimental conditions when assaying lactoperoxidase in breast milk were somewhat different. Because of the low peroxidase content of human milk, fairly large amounts, up to a maximum of 300 \( \mu l \), had to be added to the assay medium. Such large amounts of milk serum (fat-free milk) are likely to interfere somewhat with the assay due to the presence of reducing compounds like ascorbic acid and thiols. When internal standards (human milk
serum with high lactoperoxidase activity) were added to milk sera containing no or very little peroxidase activity, up to 20% inhibition was observed. It is thus possible that the milk specimens presented in Fig. 1 as possessing no or very little activity in fact contained somewhat more than that indicated.

**Bovine milk.** Cow's milk was about 20 times richer in peroxidase activity than human milk, and there was no significant decrease during the first 2 weeks after parturition (Table 2).

**Calf saliva.** Calves had a lower peroxidase activity in their saliva during the first 3 days of life than did the human infants. Later the activity increased 20-fold (Table 2).

**Treatment of milk with gastric juice.** Incubation of milk and gastric juice for 1 h at 37°C resulted in no reduction of peroxidase activity in any of the milk samples. After 4 h the enzyme activity still remained high in human milk, but there was a decline in bovine milk. No difference was seen between precolostrum and colostrum (Fig. 3). A slight decrease in activity, perhaps due to dilution, was seen in vivo when breast milk was mixed with gastric juice in the stomach for 30 min (Table 3), but there was no total inactivation.

**Thiocyanate content in human milk and saliva of newborn infants.** The concentration of thiocyanate in the saliva of newborn infants was found to be 285 ± 189 μM (mean ± standard deviation, n = 15). Human milk contained little thiocyanate (<80 μM). The exact amount could not be determined because of turbidity.

**DISCUSSION**

This study has demonstrated that human colostrum and breast milk have low lactoperoxidase activity compared with cow's milk. There is a slight decrease in enzyme activity with time after delivery. Cow's milk, on the other hand, has a rather constant amount of peroxidase during the first 2 weeks.

Saliva of newborn infants has a varying but sometimes very high lactoperoxidase activity. Thiocyanate is also present. Since the concentrations of these compounds are sufficiently large (8,11,16,24; H. Hoogendorn, Ph. D. thesis) and hydrogen peroxide can be provided by oral bacteria (11), and possibly also by oxidases in milk, the prerequisites for action of the lactoperoxidase system are present. Furthermore, lactoperoxidase in milk is not inactivated by
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gastric juice. However, the fate of the enzyme in duodenum and jejunum is not known, and in this part of the gastrointestinal tract the largely anaerobic environment also might obstruct the formation of hydrogen peroxide.

An unexplained finding is the great variability of the activity in consecutive samples from the same newborn infant, and this was not due to analytical error (see Materials and Methods). In addition, no lactoperoxidase inhibitors were demonstrated. Although, according to Table 1, there are individuals with predominantly low (cases I and II) or high (case V) activity, the variations within the same infant are great. Such variations in activity are not seen in adults (18) or children 4 to 15 years old (27). The zero values (Fig. 2) might be the result of the daily variation mentioned above, and it still is a fact that almost every newborn infant is able to produce saliva with comparatively high peroxidase activity.

The composition of saliva generally varies with the flow rate, which itself varies with the type and intensity of stimulus. The concentration of some components in saliva thus has been found to fall as the flow rate increases, but some constituents will rise in concentration when the secreted volume increases (13). There are also examples in which the composition of saliva is affected by the nature of the stimulus independently of the flow rate (B. Ahmadi-Talesch, Ph. D. thesis, Universität Düsseldorf, 1967). In this study we tried to get “unstimulated” saliva, since stimulated saliva is said to be less charac-

TABLE 1. Variation during 1 day of peroxidase activity in saliva from six newborn infants

<table>
<thead>
<tr>
<th>Infant no.</th>
<th>Peroxidase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 a.m.</td>
</tr>
<tr>
<td>I</td>
<td>4.3</td>
</tr>
<tr>
<td>II</td>
<td>1.8</td>
</tr>
<tr>
<td>III</td>
<td>9.4</td>
</tr>
<tr>
<td>IV</td>
<td>1.3</td>
</tr>
<tr>
<td>V</td>
<td>—</td>
</tr>
<tr>
<td>VI</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*Expressed as ΔA_{400} per minute per milliliter of saliva.

—, No saliva was obtained.

TABLE 2. Peroxidase activity in bovine milk and calf saliva at different times post-parturition

<table>
<thead>
<tr>
<th>Time post-parturition (days)</th>
<th>Bovine milk</th>
<th>Calf saliva</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39.8</td>
<td>4.3</td>
</tr>
<tr>
<td>3</td>
<td>47.0</td>
<td>4.6</td>
</tr>
<tr>
<td>5</td>
<td>46.0</td>
<td>14.0</td>
</tr>
<tr>
<td>14</td>
<td>34.2</td>
<td>86.2</td>
</tr>
</tbody>
</table>

*Mean of samples from three animals.

*Expressed as ΔA_{400} per minute per milliliter of milk or saliva.

Fig. 2. Lactoperoxidase activity in human saliva.
teristic of the oral cavity of an individual.

Although the salivary glands of a newborn infant are apparently histologically mature (4), there is evidence of physiological immaturity. It is not known how different stimuli like the special feeding routines at this age or our method of collecting saliva may influence the composition of saliva.

The possible biological significance of the lactoperoxidase system is debatable. A system inhibiting the growth of lactobacilli was demonstrated in cow’s milk and saliva more than 30 years ago (7,14), and lactoperoxidase was later shown to be the active factor in this system, which also inhibits several streptococci (23; H. Hoogendorn, Ph. D. thesis), Staphylococcus aureus, Escherichia coli, Candida tropicalis (11), and some viral species (3). The importance of the system in vivo has never been completely clarified in adults or in newborn infants, although some attempts have been made (H. Hoogendorn, Ph. D. thesis). Indirect evidence of its importance may be provided by the fact that nature has provided both newborn calves and humans with this system. In the former species it is provided by colostrum and to a much smaller extent by saliva (23), and in the latter the quantities are reversed. If the system has any function in this period of life, the saliva of the newborn infant must be more important than the breast milk obtained from the mother.

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LITERATURE CITED


