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Immunoglobulin D in rat serum, saliva and milk

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Summary. Previously, this laboratory has found very high concentrations of IgD in normal rat milk. Using ELISA methods, the relationship between milk, serum and saliva IgD in lactating and suckling rats was examined. Milk IgD appears to be synthesized in the mammary tissue rather than taken up from the blood because (i) serum IgD remains low and is not significantly different from that of non-lactating females, and (ii) serum IgD during lactation is poorly correlated with milk IgD. Serum IgD in suckling rats declines in the first 7 days following birth and remains relatively low during the remainder of lactation (2–4 μg/ml). The surprisingly high serum IgD observed at birth ($9{\cdot}3 \pm 3{\cdot}2$ μg/ml) is present before suckling begins and is not affected by the onset of suckling. Transient elevations of serum IgD begin to occur following weaning. Rats weaned 10 days earlier than normal (Day 20 *vs* Day 30) had significantly higher serum IgD on Days 36, 47 and 60. Among 43 adult rats, serum IgD was $5{\cdot}4 \pm 3{\cdot}6$ μg/ml and saliva IgD $2{\cdot}0 \pm 1{\cdot}7$ μg/ml. Serum IgD correlates poorly with saliva IgD. The thymus is not required for IgD synthesis since no significant difference in serum IgD was found between nude rats and their euthymic littermates.

INTRODUCTION

Although it has been more than 20 years since immunogloblin D was first discovered in a patient with multiple myeloma and in trace quantities in normal human serum (Rowe & Fahey, 1965a, b), the biological function of secreted IgD remains a mystery. IgD has been found as a major surface immunoglobulin appearing with IgM on B lymphocytes in a wide variety of species, including man (Rowe *et al.*, 1973), mouse (Abney & Parkhouse, 1974), monkey (Martin, Leslie & Hindes, 1976), rat (Ruddick & Leslie, 1977), rabbit (Sire, Colle & Bourgois, 1979), chicken (Leslie, 1980) and pig (Zikan, Sima & Tuckova, 1983). Similarly, serum IgD has been identified in monkeys (Leslie & Armen, 1974), rats (Bazin *et al.*, 1978), mice (Finkelman *et al.*, 1979) and pigs (Zikan *et al.*, 1983) in addition to humans. The evolutionary conservation of serum and membrane IgD in such diverse species itself suggests that the biological function of IgD, however mysterious, is not a trivial one. Recently, we provided preliminary evidence to suggest that IgD is a secretory immunoglobulin present in rat milk at concentrations between 50 and 300 μg/ml, based on radial immunodiffusion testing (Olson & Leslie, 1982). In this paper, we use an ELISA method to confirm these results and extend them to examine (i) the relationship between milk and serum IgD in the lactating mother and her pups, (ii) the ontogeny of serum IgD in the pup during suckling and after weaning, and (iii) levels of IgD in the saliva and serum of normal rats, and in the serum and whey of athymic nude rats.

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MATERIALS AND METHODS

Animals

HPR strain rats, bred for a high precipitin response to

group A streptococcal carbohydrate and derived from Sprague-Dawley stock, were used unless otherwise noted (Stankus & Leslie, 1974). Athymic nude rats were provided by Carl T. Hansen of NIH and were derived from hooded rats (Festing *et al.*, 1978).

Isolation of saliva, serum and milk

Anaesthetized rats were given 0·25 units of oxytocin intravenously, and milk or colostrum was collected using a vacuum pump. Milk was clarified by centrifugation at $1{\cdot}1 \times 10^5 g$ for 1 hr at 4°. Blood was collected by tail or cardiac puncture, allowed to clot at 4° overnight, and serum separated by centrifugation at 330 *g* for 15 min at 4°. Saliva was collected by suction immediately following ether anaesthesia. All samples were stored at −20° until assayed.

Preparation of rabbit anti-IgD enzyme conjugate

Heterologous rabbit anti-rat IgD was generated by immunization with membrane IgD purified from rat lymphocytes (Cuchens, Martin & Leslie, 1978). A 45% saturated ammonium sulphate precipitate of the serum was prepared and dissolved in 0·01 M Tris buffered saline, pH 7·4 (TBS). The antiserum was absorbed with whole rat serum, rat IgM and '7S' rat immunoglobulin until no detectable reaction occurred with IgM, IgG or whole rat serum by double gel diffusion. The absorbed reagent gave a strong precipitin reaction of identity between rat milk and a high-IgD rat serum assayed independently by Dr Herve Bazin, and with a second antiserum to IgD raised in sheep. IgD-specific antibodies were isolated from an immunoadsorbant made with partially purified milk IgD (see below). After further gel diffusion tests to prove specificity, the antibodies were coupled to alkaline phosphatase (Sigma type VII-S) (Kearney *et al.*, 1979). The enzyme conjugate bound to vinyl plates coated with partially purified IgD, but not to rat IgM, '7S' salt-precipitated immunoglobulin or IgA-coated plates. Demonstration of specificity was shown further by lack of binding inhibition by rat IgM, IgG2c, '7S' salt-precipitated immunoglobulin, IgA, or antiserum to IgA and the presence of binding inhibition by partially purified IgD and a high-IgD rat serum. Human myeloma sera and murine myeloma ascites fluid with high levels of IgD (the mouse ascites kindly provided by Dr Fred Finkelman) showed some binding inhibition, while IgD-deficient human and mouse serum did not. This supports studies indicating the presence of cross-reactivity between rat, human and mouse IgD (Golding *et al.*, 1979).

Total IgD assay

Ninety-six well vinyl assay plates (Costar no. 2095) were coated for 2 hr with 1 μg/ml partially purified IgD in pH 9·6 carbonate-bicarbonate buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 3 mM sodium azide). The plates were rinsed once with pH 7·0 10 mM phosphate-buffered saline (PBS), and unreacted sites blocked with 1% BSA in PBS for 2 hr at 20°. The plates were rinsed four times with PBS containing 0·05% Tween 20 (Sigma, St Louis, MO) and four times with PBS. Serum to be assayed was diluted in 2% normal rabbit serum/PBS, mixed with the rabbit anti-rat IgD enzyme conjugate, and incubated in wells for 2 hr at 20°. Plates were rinsed as before. Finally, 0·5 mg/ml *p*-nitrophenyl phosphate (Sigma 102) in 1 M diethanolamine, 1 mM $MgCl_2$ (pH 9·8) was added to the plates, incubated for 1 hr at 37°, and read on an ELISA plate reader. A pooled sample of rat milk containing 120 μg/ml IgD (based on comparison with a high-IgD rat serum assayed by Dr Bazin) was used as the IgD standard.

Total protein assay

Total protein was measured by optical density at 280 nm of the clarified milk samples ($r = 0{\cdot}99$ *vs* protein concentration as assayed by the Bradford method (Bradford, 1976)).

Partial purification of milk IgD

Whole rat milk was passed over a $2{\cdot}5 \times 90$ cm Sephacryl 300 gel filtration column (Pharmacia, Piscataway, NJ) equilibrated with pH 7·4 TBS. The IgD-rich fractions were determined by Ouchterlony analysis, then pooled and concentrated.

RESULTS

Quantitation of IgD in milk, serum and saliva from lactating rats

Seven lactating rats were milked every other day from Day 5 to the end of lactation (Day 30). The IgD concentration in milk ranged from 36 to ~800 μg/ml. Milk IgD ranged from 100 to 300 μg/ml between Days 5 and 21. After Day 21, it tended to increase (Fig. 1). The variation in the levels of IgD in milk mainly appears to be the result of individual differences in milk output versus IgD output. The end-of-lactation increase was accompanied by a parallel increase in milk protein (Fig. 1). Milk IgD (μg/mg of clarified

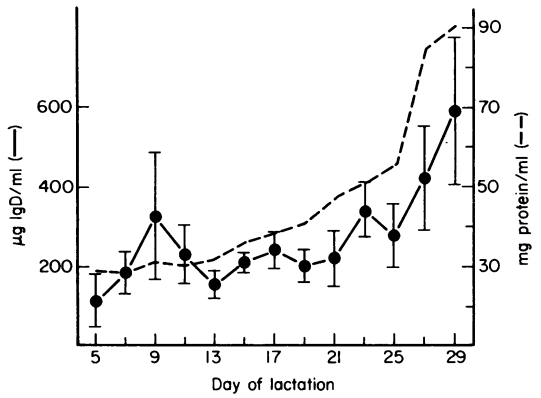

Figure 1. IgD concentration (●– – –●) and protein concentration (– – – – –) of whey during lactation. Values are mean ± SD.

milk protein) varied between 5 and 11 μg/mg, with some rats experiencing an increase around Day 9 as reflected in the mean (Table 1). Serum IgD in lactating females showed considerable variability, but did not differ overall from values seen in non-pregnant and non-lactating rats. Milk IgD concentration (μg/mg clarified milk protein) and serum IgD concentration correlated very poorly ($r=0{\cdot}13$, $n=40$). Fifteen samples of saliva from five other lactating rats had a mean IgD of 0·9 ± 0·7 μg/ml.

Table 1. Serum and whey IgD concentration during lactation

Day of lactation	Whey IgD* (μg/mg whey protein ± SD)	Serum IgD (μg/ml ± SD)
5	6·6 ± 2·8 (4)†	4·5 ± 2·0 (4)
7	7·8 ± 5·1 (6)	5·6 ± 2·7 (3)
9	10·3 ± 9·2 (7)	4·3 ± 1·4 (3)
11	9·7 ± 6·3 (7)	10·5 ± 8·6 (4)
13	6·0 ± 2·4 (5)	5·1 ± 3·9 (4)
15	7·5 ± 1·7 (6)	4·5 ± 2·9 (4)
17	7·0 ± 2·3 (7)	5·2 ± 2·5 (7)
19	5·8 ± 3·1 (7)	5·4 ± 2·0 (5)
21	6·0 ± 3·7 (7)	10·8 ± 10·6 (5)
23	6·9 ± 3·5 (7)	3·6 ± 0·9 (5)
25	6·7 ± 3·7 (5)	6·2 ± 5·1 (4)
27	6·4 ± 2·7 (3)	2·1 ± 0·5 (2)
29	5·9 (1)	6·0 ± 1·0 (2)

* μg IgD/mg whey protein was determined for each lactating rat, and then the mean ± SD calculated.

† The number of rats in each sample is given in parentheses.

IgD in suckling and weaned rats

Serum was collected from suckling and weaned rats between birth and 66 days of age. One litter followed between Days 1 and 42 showed a decline of > 67% in their serum IgD between Day 1 and Day 7 of lactation (from 9·3 μg/ml to 2·8 μg/ml), remaining at between 2 and 4 μg/ml until the end of lactation at Day 30, when the serum IgD rose sharply in some of the rats, and then declined towards Day 42. A group of August rat sera assayed between Day 21 and Day 66 had serum IgD levels of ~1 μg/ml during the last ten days of lactation, rose to 2·7 μg/ml at Day 30, then declined to 1–2 μg/ml by Day 66 (Table 2). A third group ($n=20$) from three litters was followed from Day 1 to Day 60. In the first 8 days of suckling, serum IgD declined from 6·3 μg/ml to 1·8 μg/ml, then remained at 1·8–2 μg/ml to the end of lactation, when the mean level rose owing to transient elevations among individual rats (Table 3).

The relatively high levels of serum IgD consistently observed in newborn rats did not appear to be the result of haemoconcentration, since radial immunodiffusion assays of serum transferrin and albumin did not show a similar decline during the first 7 days of life

Table 2. Ontogeny of serum IgD in HPR and August rats*

Age (days)	Concentration of IgD (μg/ml ± SD) (HPR rats)	(AUG rats)
1	9·3 ± 3·2 (4)†	–
4	5·5 ± 1·4 (4)	–
7	2·8 ± 1·1 (6)	–
10	3·4 ± 1·7 (6)	–
14	2·4 ± 1·3 (11)	–
17	3·6 ± 0·8 (10)	–
21	2·8 ± 0·9 (5)	1·0 ± 0·6 (4)
24	1·9 ± 1·7 (5)	–
28	2·6 ± 2·2 (5)	0·9 ± 0·3 (6)
30	–	2·7 ± 1·4 (6)
32	4·5 ± 3·5 (4)	–
36	10·6 ± 8·9 (4)	–
38	–	1·7 ± 0·5 (5)
42	5·7 ± 2·7 (4)	–
48	–	1·6 ± 1·1 (5)
60	–	1·4 ± 0·6 (5)
66	–	1·1 ± 0·5 (4)

* Rats were allowed to wean naturally at 30 days of age.

† Numbers of individual sera tested are given in parentheses.

Table 3. Influence of early weaning on serum IgD*

Day		Pup serum IgD (μg/ml ± SD)	Maternal serum IgD (μg/ml ± SD)	Whey IgD (μg/ml ± SD)
1		4·2 ± 1·4 (9)†	–	–
8		1·8 ± 0·8 (18)	6·3 ± 4·8 (2)	189 ± 170 (3)
15		1·9 ± 0·7 (19)	2·0 ± 0·2 (2)	237 ± 75 (2)
20		1·0 ± 0·4 (19)	2·6 ± 1·0 (3)	213 ± 137 (3)
24	E:	3·1 ± 3·4 (9)		
	L:	1·4 ± 0·3 (9)	3·1 ± 2·6 (3)	200 ± 36 (3)
	$\bar{x}$:	2·3 ± 2·5 (18)		
28	E:	1·8 ± 1·2 (9)		
	L:	1·3 ± 0·7 (10)	2·7 ± 0·8 (3)	
	$\bar{x}$:	1·5 ± 1·0 (19)		
32	E:	7·2 ± 9·9 (8)		
	L:	2·1 ± 1·2 (10)	2·6 ± 0·5 (3)	
	$\bar{x}$:	4·4 ± 6·9 (18)		
		(P = 0·12)‡		
36	E:	12·1 ± 8·5 (6)		
	L:	1·9 ± 0·5 (5)	9·3 ± 7·7 (2)	
	$\bar{x}$:	7·5 ± 8·0 (11)		
		(P = 0·04)		
40	E:	2·4 ± 4·0 (8)		
	L:	2·8 ± 4·1 (8)	5·7 ± 2·5 (2)	
	$\bar{x}$:	2·6 ± 3·9 (16)		
47	E:	30·9 ± 82·9 (9)		
	L:	1·3 ± 0·3 (7)	2·1 (1)	
	$\bar{x}$:	17·9 ± 62·5 (16)		
		(P = 0·01)		
60	E:	12·1 ± 28·8 (9)		
	L:	1·6 ± 0·2 (8)		
	$\bar{x}$:	7·2 ± 21·1 (17)		
		(P = 0·004)		

* Half of three litters of rats were weaned at 20 days of age, the rest allowed to wean naturally at about Day 30. The means for the resulting groups are designated 'E' and 'L' respectively; '$\bar{x}$' is the mean for all the rats in both groups.

† Numbers of individual sera tested are given in parentheses.

‡ P values represent the probability that the difference between the E and L groups are not statistically significant, based on the Mann–Whitney U-test.

but instead showed a gradual increase (data not shown). Since IgD in colostrum is high on Day 1 of lactation (Olson & Leslie, 1982), it seemed reasonable to suspect that high serum IgD at birth represented a brief period of transport of milk IgD into suckling rat serum. In order to test for this, serum IgD was assayed in serum taken from half a litter of eight newborn rats on Day 1 before suckling had begun, and in serum taken from the remaining half after these rats had suckled for a day.

The Day 1 unsuckled rats had a mean serum IgD of 8·5 μg/ml (SD = 3·9) and the Day 2 suckled rats had a mean serum IgD of 7·9 μg/ml (SD = 0·7), which indicates that the first day of suckling had no apparent positive influence on suckling rat serum IgD. Thus, high serum IgD observed in newborn rats is acquired either transplacentally or is synthesized *de novo* by the newborn rat. This does not exclude the possibility that milk IgD makes a contribution to serum IgD in the newborn rat.

If the serum IgD observed in newborn rats were synthesized by the newborn rat and not acquired from

the mother via the placenta, then one hypothesis which could explain the low serum IgD in suckling rats is that ingestion of milk suppressed serum IgD. Therefore, weaning rats 10 days earlier than normal might result in the earlier appearance of elevated serum IgD in some of the weanlings. In order to test this, rats from three litters were bled periodically between Day 1 and Day 60. At Day 20, half of each litter was weaned and the other half left to suckle naturally for an additional 10 days.

Each group had an equal number of males and females. No significant difference between the groups was observed on Day 24 and Day 28 following early weaning, hence there was no evidence to suggest that early weaning resulted in the earlier appearance of transiently elevated IgD (Table 3). However, the early-weaned rats showed a distinctly greater tendency to develop transiently elevated serum IgD of a greater magnitude than that of the late-weaned rats. A highly significant difference in serum IgD was observed on Days 36, 47 and 60, with a marginal difference observed on Day 32. On days 47 and 60, one rat in the early-weaned groups showed a serum IgD level of 252 μg/ml and 88·6 μg/ml, respectively. Of the 13 serum samples which had >6 μg/ml IgD (200 tested), one was from the late-weaned group and twelve were from the early-weaned group.

Serum and saliva IgD in adult rats

Normal adult rats ($n=43$) with a mean age of 319 days had $5{\cdot}4 \pm 3{\cdot}6$ μg/ml serum IgD and $2{\cdot}0 \pm 1{\cdot}7$ μg/ml of saliva IgD. The 20 female rats with a mean age of 304 days had somewhat more serum IgD than 23 male rats with a mean age of 340 days ($7{\cdot}1 \pm 3{\cdot}5$ μg/ml *vs* $4{\cdot}1 \pm 3{\cdot}1$ μg/ml). Saliva samples taken from 19 of the female rats had $1{\cdot}9 \pm 1{\cdot}9$ μg/ml IgD, while 15 of the male rats had $2{\cdot}2 \pm 1{\cdot}4$ μg/ml. No significant correlation was found between serum IgD and age ($r=0{\cdot}17$), saliva IgD and age ($r=0{\cdot}09$), or saliva and serum IgD ($r=0{\cdot}09$). In adult rats as in young weaned rats, the level of serum IgD is highly variable from individual to individual. Although no longitudinal study was done in this group of rats, tests on other individual sera at different times indicate that, as with younger rats, serum IgD can fluctuate considerably over time in any one animal (data not shown).

A group of six 68-day-old nude rats had $5{\cdot}0 \pm 1{\cdot}1$ μg/ml serum IgD, whereas their six euthymic heterozygous littermates had $3{\cdot}9 \pm 1{\cdot}0$ μg/ml. A second group of six 72-day-old nude rats had $2{\cdot}7 \pm 2{\cdot}6$ μg/ml serum IgD, whereas 19 euthymic (rnu/+ and +/+) littermates had a mean serum IgD of $1{\cdot}4 \pm 0{\cdot}9$ μg/ml. These results confirm the findings of Bazin *et al.* (1980), in that serum IgD in nude rats is not significantly different from euthymic littermates, and indicates that serum IgD does not require the thymus for the maintenance of normal levels. The same appears to be true for IgD production in milk. Milk obtained on Day 2 from a lactating athymic nude rat contained 232 μg/ml IgD, which was not markedly different from that observed in normal lactating rats.

DISCUSSION

High levels of IgD exist normally in rat milk (Olson & Leslie, 1982). Two questions concerning milk IgD are (i) whether IgD occurs in milk as a transudate from serum or is genuinely a secretory immunoglobulin, and (ii) where milk IgD finds its site of action within the suckling pups. In cattle, Dixon, Wiegle & Vanquez (1961) showed that a prepartum drop in serum IgG1 was accompanied by the selective transport of that immunoglobulin into milk (for review, see Butler, 1983). More recently, Halsey *et al.* (1982) showed that significant changes take place in serum polymeric IgA and in serum albumin in lactating mice. Thus, if milk IgD is the result of transudation from serum, then the start and end of lactation might well be expected to be accompanied by definite changes in serum IgD, especially in light of the roughly one-hundred fold difference in milk and serum IgD concentrations. For milk IgD to be transported from serum without such changes, it would be necessary (i) that the rates of synthesis and mammary uptake complement one another exactly, and (ii) that any non-secretory function of serum IgD be either disrupted or be protected by a mechanism which distinguishes it from secreted IgD. Since there is a poor correlation between serum IgD and milk IgD, and because there is no significant difference between levels of serum IgD between lactating and non-lactating rats, it appears more likely that milk IgD is secreted by cells in the mammary tissue as a genuine secretory immunoglobulin, and that it is regulated independently of serum IgD. Proof of this hypothesis awaits histological or radioactive tracer studies. However, it is interesting to note that IgD levels in human milk also correlate poorly with those of serum IgD (Bahna, Keller & Heiner, 1982), and histological studies of IgD-containing cells in human mammary tissue are consistent with a local synthesis

model rather than a transudation model for milk IgD (Brandtzaeg, 1983).

Newborn rats possess high levels of serum IgD before suckling, thus their serum IgD is either synthesized by themselves or acquired from the mother via the placenta. Since serum IgD does not change after a day of suckling and declines in the first week, any contribution of milk IgD to serum IgD is not enough to reverse its decline there. If IgD in milk does pass into the circulation of the suckling rat, it may be an inefficient process, quickly sequestered from the blood, rapidly redistributed to other secretory sites, or catabolized.

There are a number of possible explanations for the decline in serum IgD following birth. One explanation is that the serum IgD observed on Day 1 is acquired via the placenta and is gradually catabolized following birth. Another possibility is that serum IgD in the newborn rat is synthesized by the newborn rat itself and that something in milk (IgD?) acts to suppress its synthesis. However, while weaning had the effect of increasing the magnitude of transient elevations of serum IgD, it did not cause the elevations to begin appearing earlier, which argues against such a suppressive mechanism transmitted by milk.

The significantly higher serum IgD observed in rats weaned 10 days early could be the result of either premature cessation of suckling or the premature exposure of the gut to solid food antigens. The stress of early weaning could have induced the effect, however both groups of rats continued to gain weight at a comparable rate and were in apparently good health until the end of the study.

The cause of elevated serum IgD is not known. In clinical studies, elevated IgD has been associated with a variety of diseases and immunological disorders (for review, see Leslie & Martin, 1977), but it can also appear in apparently healthy individuals. Elevated serum IgD in our rat studies was not associated with any overt disease. As elevations in serum IgD occur in apparently healthy rats and occur as a transient rather than chronic state, such phenomena appear to be a consequence of normal immune function. While it is not known whether the elevations represent specific immune responses or non-specific events, Pauwels *et al.* (1979) have shown that immunization of rats with ovalbumin with *B. pertussis* and aluminum hydroxide results in a 'non-specific' increase in serum IgD. The somewhat higher level of serum IgD in female rats may reflect an association of serum IgD with the generally higher immune responsiveness among female rats. Since nude rats can develop normal levels of both milk and serum IgD, and can develop elevated serum IgD with at least the same frequency as their heterozygous or non-nude littermates, it is clear that T-cell help is not required for the production of IgD in serum or milk.

An important question which remains is that of the antigen specificity of IgD. A very limited number of clinical studies have gone beyond measuring total IgD levels to detect or measure antigen-specific IgD. The unavailability of an animal model until the last few years has been an obstacle to systematic study. Pauwels *et al.* (1979) reported that in rats a small amount of IgD anti-ovalbumen could be elicited by intraplantar immunization with ovalbumin and adjuvants.

Owing to the high concentration of IgD in milk, it appeared feasible to induce measurable IgD immune responses in milk by intramammary immunization. Our preliminary results indicate that IgD antibodies to haptenated protein can be induced in both milk and serum by such an immunization protocol. Currently, we are engaged in efforts to characterize further this immune response and to use it as a probe for defining more precisely the sites of IgD synthesis and sites of potential transport in both the mother and the suckling rat.

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