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Ontogeny of the secretory immune system: Maturation of a functional polymeric immunoglobulin receptor regulated by gene expression

(secretory component/hepatocytes/mRNA/developmental process)

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ABSTRACT **In the rat, secretion of polymeric IgA from serum into bile is dependent upon the presence of a functional polymeric immunoglobulin receptor (pIgR) that acts as a hepatocyte plasma membrane receptor for ligand binding and as a transcellular transport molecule. The objective of this study was to document the developmental maturation and regulation of functionally intact rat liver pIgR. An adult pattern of IgA secretion was not detected until after day 23 postpartum (dPP), by using intravenously injected ^{125}I-labeled dimeric IgA. Radioactive dimeric IgA was not detectable in hepatocyte transport vesicles until 21 dPP by electron microscopy autoradiographic analysis. By using a rabbit polyclonal antibody against the rat secretory component domain of the pIgR, Western blot analysis demonstrated that the plasma-membrane-bound pIgR levels in hepatocytes from rats aged 19–22 dPP increased 10-fold during this period. To determine whether or not this increase in membrane-bound pIgR reflected increased pIgR gene expression, we probed Northern blots of total cellular RNA extracted from neonatal rat liver with pIgR cDNA [GORF-1; Banting, G., Brake, B., Braghetta, P., Luzio, J. P. & Stanley, K. K. (1989) *FEBS Lett.* 254, 177–183]. The pIgR RNA levels between 19 and 22 dPP rose more than 20-fold and paralleled the increased membrane-bound pIgR protein during this same interval. These data demonstrate a developmentally regulated process that controls the ontogeny of biliary dimeric IgA secretion at the termination of the third week postpartum. The process appears to depend on the up-regulation of pIgR gene expression.**

In rats, circulating polymeric immunoglobulins are transported across hepatocytes to the bile by a sinusoidal plasma membrane (SPM) polymeric immunoglobulin receptor (pIgR) (1). The primary structure of the pIgR is known (2, 3), and portions of this molecule are fairly well conserved in humans, rabbits, and rats (4–6). There is a large extracellular poly-immunoglobulin binding domain [secretory component (SC)], a membrane-spanning hydrophobic domain (membrane anchor), and a hydrophilic cytoplasmic tail. The pIgR is a glycoprotein synthesized on polysomes attached to the rough endoplasmic reticulum, translocated across the endoplasmic reticulum membrane (105 kDa), and terminally glycosylated in the Golgi complex to the transmembrane form (116 kDa) (7, 8). After phosphorylation of the serine at position 664 in the cytoplasmic tail (9) to 120 kDa (10) and binding its ligand [dimeric IgA (dIgA)], the resulting complex is internalized into endocytic vesicles and transcytosed to the bile canalicular plasma membrane (11–13). A portion of the pIgR is cleaved (14) and secretory IgA is released into the bile. Free secretory component (FSC) will be released into the bile if the receptor is transcytosed without its ligand (15–17).

Newborn rodents have an immune system that is relatively immature. For example, the intestinal secretory immune system in the rat does not mature until approximately 3 weeks postpartum. At this time, there is enhanced intestinal crypt cell proliferation, intestinal plasma cell production, and SC (pIgR) expression (18, 19). In rats, pIgR is synthesized in intestinal crypt cells and hepatocytes. Polymeric IgA, the most abundant immunoglobulin in external secretions, is produced by the plasma cells.

Although the sorting and targeting steps of the pIgR pathway (9, 17, 20–24) and the location within the hepatocyte of the cleavage event (14, 17, 25–27) have been subjected to considerable study, little is known about the developmental pattern of either polymeric IgA transport or pIgR expression on the hepatocyte plasma membrane. Our data suggest that the timing is coincident for three events: (*i*) the appearance of pIgR on the SPM, (*ii*) the maturation of the functional receptor as shown by efficient dIgA transport, and (*iii*) the increase of steady-state levels of mRNA for pIgR. The data support the existence of a developmental process involving up-regulation of pIgR gene expression at the termination of the third week postpartum.

MATERIALS AND METHODS

Animals. Fischer 344 rats [Bantin & Kingman (Fremont, CA) or Simonsen Laboratories (Gilroy, CA)] were housed at the Department of Veterans Affairs Medical Center Animal Research Facility and allowed food and water ad libitum. The pups were left with nursing mothers until the day of sacrifice. All animals were maintained in accordance with guidelines of the Animal Studies Subcommittee of the Veterans Affairs Medical Center.

Radioisotopes and Other Chemicals. [^{125}I]NaI and [^{32}P]dCTP were purchased from Amersham. Guanidinium isothiocyanate was obtained from Fisher, phenol was from BRL, rat IgG was from Cappel Laboratories, and all other chemicals were from Sigma.

IgA Transport Studies. The dIgA myeloma IR699 protein (28) (gift of H. Bazin, University of Louvain, Brussels) was purified from ascites fluid by HPLC on a Spherogel TSK 3000SW column (Beckman Instruments) in 0.1 M $KHSO_4$ and iodinated using a modification of the monochloride method (29) to a specific activity of 0.1–0.3 μCi/μg (1 Ci = 37 GBq).

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Abbreviations: pIgR, polymeric immunoglobulin receptor; SC, secretory component; dIgA, dimeric IgA; dPP, day(s) postpartum; FSC, free secretory component; SPM, sinusoidal plasma membrane.

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The rat dIgA purified in this way does not bind to the asialoglycoprotein receptor (24, 30). The pups were anesthetized with i.p. administered Nembutal [8.3 mg/ml, 0.1 ml/100 g (body weight)]. The common bile duct of each animal was cannulated with a pulled-glass pipet, matched to the size of each individual bile duct. The ^{125}I-labeled dIgA (5 μg per animal) was injected into the femoral vein. After the injection of IgA, bile was collected serially from the cannulated common bile duct for 10- or 20-min intervals over a total period of 90 min. Bile was collected on ice in the presence of protease inhibitors (aprotinin at 2 μg/ml, leupeptin at 2 μg/ml, pepstatin at 2 μg/ml, soybean trypsin inhibitor at 4 μg/ml, and phenylmethylsulfonyl fluoride at 50 μg/ml). Radioactivity in total bile samples was measured using a Beckman model 8500 γ counter.

Autoradiography. The animals were anesthetized as above, and 30 min after i.v. injection of ^{125}I-labeled dIgA into the femoral vein, the animals were perfused through the portal vein with fixative [2.8% (vol/vol) glutaraldehyde/0.8% paraformaldehyde in 0.1 M sodium bicarbonate (pH 7.4)]. The liver was removed and cut into 1-mm^3 pieces. After 2 h in the above fixative, the pieces were rinsed overnight in 0.1 M bicarbonate buffer, post-fixed in 2% (wt/vol) reduced osmium tetroxide, and prepared for electron microscopic autoradiography as described (12).

Antibody Production. A rabbit polyclonal antibody was prepared against FSC obtained from rat bile and purified by IgM affinity chromatography and Sephadex G-200 chromatography as described (31). The antibody was precipitated from rabbit serum with 50% saturated ammonium sulfate, dialyzed against 10 mM sodium phosphate (pH 7.4), and purified over a protein A column (Sigma). The antibody reacted with the FSC and membrane-bound pIgR and was absorbed vs. rat spleen cells (to reduce nonspecific binding) before use in immunoblot analyses.

Isolation of SPM. SPMs were purified from rats of various postnatal ages as described (32) in the presence of protease inhibitors (aprotinin at 1 μg/ml, leupeptin at 1 μg/ml, soybean trypsin inhibitor at 10 μg/ml, benzamidine at 1.6 μg/ml, and 1 mM phenylmethylsulfonyl fluoride). Membrane protein concentration was determined by the Bradford method (33). SPM samples were frozen at −70°C.

Immunoblot Analysis of pIgR on SPM. SPM protein (15 μg) was solubilized and reduced by boiling for 2 min in 0.5 M Tris·HCl (pH 6.8) containing 2% (wt/vol) SDS (final concentration), 10% (vol/vol) glycerol, 5.0% (vol/vol) 2-mercaptoethanol, and 0.05% bromphenol blue. The mixture was analyzed on SDS/7.5% polyacrylamide gels (34), and electrophoresis was performed at 100 mA for 2 h in a Bio-Rad Mini Protean cell at 4°C. After electrophoresis the proteins were transferred (35) onto Immobilon sheets (Millipore) in a Bio-Rad Mini Transblot apparatus at 100 mM for 1 h at room temperature. The Immobilon sheet was blocked overnight with 5% (wt/vol) nonfat dried milk (Lucerne) in 10 mM Tris·HCl (pH 7.6) and then incubated for 1 h with polyclonal rabbit anti-rat FSC (4 μg/ml) in 10 mM Tris·HCl, pH 7.6/5% nonfat dried milk. After washing in 10 mM Tris·HCl, pH 7.6/0.1% Tween 20, peroxidase-conjugated protein A (1:8000 dilution; Sigma) was added in the same buffer for 1 h at room temperature with agitation. The blot was rinsed and visualized by luminescence detection (ECL System; Amersham) according to the manufacturer's instructions. The lower limit of detection is 5 ng of pIgR protein.

RNA Purification. All solutions and glassware were treated with 0.1% diethyl pyrocarbonate (36). Total cellular RNA was isolated from 1 g of rat liver according to the method of Chomczynski and Sacchi (37). In brief, the tissue was homogenized in 4 M guanidinium isothiocyanate lysis buffer (37), extracted with phenol/chloroform, and precipitated at −20°C with isopropanol. Total RNA (35 μg) was resolved on a 1% agarose gel containing 2.05 M formaldehyde, 2.6× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate, pH 7.0), and 0.02 M Mops (free acid, molecular biology grade). The RNA was transferred to a nylon membrane (Hybond-N$^+$; Amersham) by capillary blotting overnight at room temperature using 20× SSC and UV cross-linked (38).

Probes. The rat rRNA probe used was obtained from plasmid pXC-1. We used the 5.6-kilobase (kb) *Hin*dIII fragment cloned in pBR322 (39). The pIgR cDNA probe was obtained from the GORF-1 plasmid (4). We used the 2.3-kb *Eco*RI fragment cloned in pUEX containing most of the coding sequence plus all of the 3′ noncoding sequence.

Radiolabeling of cDNA Probe. Rat pIgR cDNA (50 ng) from the plasmid GORF-1 was labeled with [^{32}P]dCTP by random priming (Amersham, according to the supplier's instructions). The labeled probe was purified over a Sephadex G-50 column to remove unincorporated nucleotides. The rRNA probe was labeled and purified by the same method.

Hybridization. After prehybridization in 3× SSC/50% (vol/vol) formamide/denatured salmon sperm DNA (0.4 mg/ml)/0.1% SDS/5.0% (wt/vol) dextran sulfate in 5% Denhardt's solution (3 h at room temperature), the blots were hybridized in the same buffer with ^{32}P-labeled pIgR cDNA probe (specific activity, 1.6 × 10^9 cpm/μg of DNA, 10^6 cpm/ml of hybridization solution) at 42°C for 30 h. The blots were washed sequentially with 2× SSC, 0.5× SSC, and 0.1× SSC (all containing 0.1% SDS) at 60°C for 45 min per wash. The blots were exposed to XAR film (Kodak) for autoradiography overnight at −70°C with one intensifying screen.

RESULTS

Onset and Pattern of IgA Transport. A major objective was to determine the timing of efficient pIgA transport across the hepatocyte. The blood to bile transport of ^{125}I-labeled dIgA injected into the femoral vein was monitored in rats at various postpartum ages (Fig. 1). This laboratory has shown (12, 13) that transport peaks at 40 min and may reach 60% of the injected dose in adult Fischer 344 rats. At early postpartum intervals [e.g., 10–15 days postpartum (dPP); data not shown], there was minimal transport (≤1%) during the 90-min period of bile collection. This may be indicative of either

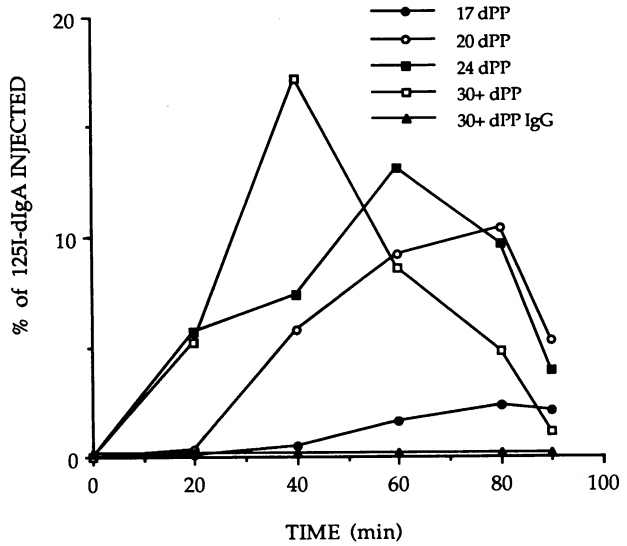

FIG. 1. Transport of ^{125}I-labeled dIgA (125I-dIgA). Data are shown for rat siblings at various postpartum ages. The animals were injected with 5 μg of ^{125}I-labeled dIgA in the femoral vein and bile samples were collected for 10- or 20-min periods over a total of 90 min. The data were plotted as percent of the total injected dose transported vs. time.

passive transport or intercellular leakage. A low rate of transport that steadily increased with the experimental time but did not peak appeared by 17 dPP. These data showed that the rat hepatocyte at 17 dPP had the ability to transport dIgA, although at a very low level. Between 17 and 20 dPP, the amount of IgA transported increased but did not peak. By 24 dPP, the total amount transported increased and exhibited a definite peak at 60 min. Between 24 dPP and adult (35 dPP in this experiment), the transport of dIgA exhibited an adult pattern with a sharp peak at 40 min. Siblings were used in each experiment to eliminate litter differences, which we found could shift the pattern ±1 or 2 days. The pups remained with the mother until 24 dPP to eliminate the influence of weaning. The transport of radiolabeled rat IgG was used as a control. Even in the adult, no significant transport of IgG occurred, indicating that this phenomenon of active transport is specific for the pIgR–dIgA complex. This also indicated that the low level of ^{125}I-labeled dIgA found in the young (15–17 dPP) was actually specific transport and not leakage or nonspecific immunoglobulin transport.

The pattern of total ^{125}I-labeled dIgA recovered in the bile is shown in Fig. 2 (mean ± SEM at various ages). Low levels of transport are seen up to 17–19 dPP, followed by a 3-fold increase at 20 dPP. An adult level of transport is reached at 22 dPP.

Electron Microscopy. In the adult rat, dIgA is transported across the hepatocytes in endocytic vesicles (13). By using electron microscopic autoradiography, we tested the developing hepatocyte's ability to bind the dIgA at the sinusoidal surface and its ability to transport the bound ligand in vesicles toward the bile canaliculus. We observed no accumulation of ^{125}I-labeled dIgA grains at the SPM, eliminating the possibility that the receptor was binding at the plasma membrane but not able to transcytose. Furthermore, no radioactive grains were observed within the intracellular vesicles until after 19 dPP.

Developmental Expression of pIgR on SPM. Vaerman *et al.* (18) have shown that the *in vivo* and *in vitro* expression of rat SC in total rat liver homogenates and cultured hepatocytes starts at 20 dPP and continues to increase to 40 dPP. In the present study, a standard amount of isolated liver SPM protein (15 μg) from rats of various postpartum ages was loaded on 7.5% polyacrylamide gels and analyzed on an immunoblot using polyclonal rabbit anti-rat SC (Fig. 3). The pIgR bands were located at approximately 116 and 120 kDa. In the lanes marked 15–17 dPP, there was a minimal amount of pIgR. The amount of pIgR was slightly higher at 18 dPP and increased dramatically at 19–21 dPP. This increased pIgR expression was confirmed by densitometric analysis using the SCAN ANALYSIS (Biosoft) program, showing by 20 dPP a 5.5 times increase in pIgR levels over 17 dPP (data not shown).

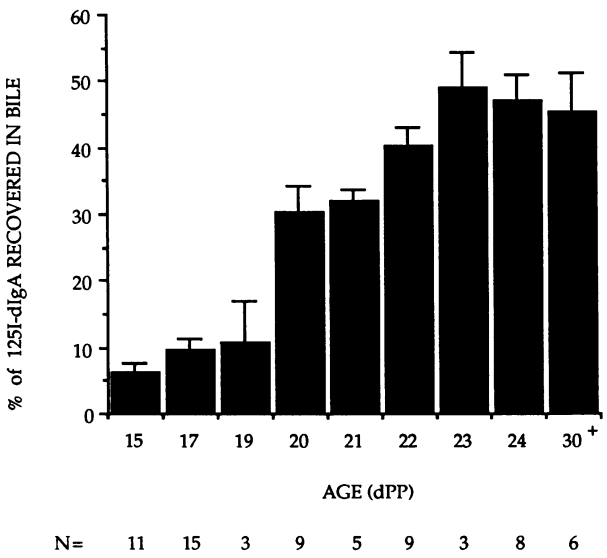

FIG. 2. Experimental procedures are the same as in Fig. 1. Data are presented as the mean ± SEM of total ^{125}I-labeled dIgA (125I-dIgA) collected over 90 min (N = the number of animals at each postpartum time period pooled from several litters).

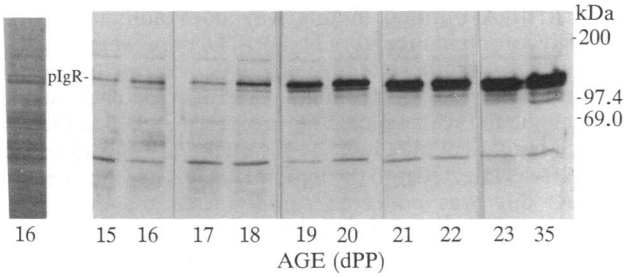

FIG. 3. Developmental pattern of pIgR expression on isolated liver plasma membrane preparations of rat siblings. SPM proteins (15 μg) obtained at the various postpartum ages (indicated by the numbers at the bottom of the lanes) were loaded on 7.5% polyacrylamide gels, transferred to Immobilon, and probed with polyclonal rabbit anti-rat SC and protein A-peroxidase. The immunoreactive bands were visualized using the ECL system. The positions of molecular mass markers are indicated on the right (Amersham rainbow markers). Lane on the far left shows a Coomassie blue stained gel of total SPM protein.

Age-Related Changes in pIgR mRNA. The increased transport of dIgA and increased membrane-associated pIgR protein in 21-day-old rats suggests that pIgR gene expression increases during the 19- to 22-dPP period. If this is true, it should be possible to see a parallel increase in the level of pIgR mRNA during this same time frame. GORF-1 plasmid was obtained containing a 2.4-kb cDNA insert of the rat pIgR gene (4). This insert contains a 1.5-kb sequence with homology to the human and rabbit pIgR coding sequences and a 0.9-kb section of 3′ noncoding sequence homologous to the corresponding rabbit cDNA. As a preliminary experiment, the 2.3-kb *Bam*HI fragment of the GORF-1 insert was used as a probe of Northern blots of total RNA isolated from a 90-day-old rat and from neonatal rats of 5 and 15 days of age. The results are illustrated in Fig. 4*A*. The GORF-1 probe specifically hybridized to a predominant mRNA of about 4.0 kb. The levels of this mRNA in both 5- and 15-day-old neonatal liver are less than 10% that of the adult rat. Confirmation of even loading of RNA is shown in Fig. 4*B*, the ethidium bromide staining of the same blot. Since the trans-

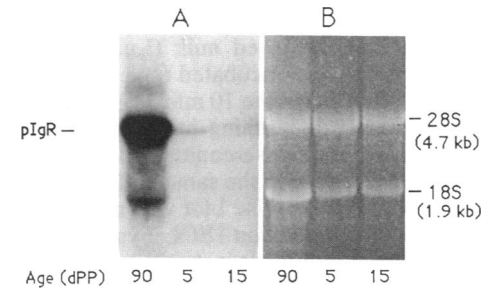

FIG. 4. pIgR gene expression in adult and neonatal liver. Total RNA isolated from rat livers of the indicated ages was loaded and electrophoresed on a 1% agarose/formaldehyde gel. Total RNA (35 μg) was loaded per lane. (*A*) Hybridization of ^{32}P-labeled pIgR cDNA (GORF-1) to total RNA. (*B*) Ethidium bromide stain of the same agarose gel. The intensity of the rRNA bands in each lane confirms that comparable amounts of RNA were loaded onto each lane.

port data and Western blot analysis indicated that an increase in dIgA transport and SPM–pIgR levels occurred between 19 and 22 dPP, we tested for a parallel increase in pIgR mRNA during the same period. Fig. 5 shows the results of a Northern blot analysis using total cellular RNA isolated from rat littermates of different ages ranging from 18 to 28 dPP. The blot was probed with both GORF-1 and a DNA probe homologous to the rat 18S rRNA (39) to more stringently control for RNA loading. Fig. 5*A* shows the blot probed with pIgR cDNA (GORF-1), indicating very low levels of specific mRNA until 20 dPP. Then at 21 dPP the level increases dramatically and continues to increase until 25–28 dPP. Fig. 5*B* shows even loading of RNA at all ages. Laser densitometry of the GORF-1-positive band (mRNA specific) and 18S rRNA homologous bands was performed. The values of the GORF-1 bands were normalized to that of the 18S rRNA bands and expressed as a ratio of band intensities (Fig. 5*C*). The results show that during the period between 19 and 22 dPP there is a significant increase (>20-fold) in the levels of GORF-1 homologous mRNA that closely corresponds to the observed increases in SPM–pIgR protein and pIgA transport into bile.

DISCUSSION

Our data provide evidence that SC production is developmentally regulated in rat hepatocytes. Within the narrow time period of 19–22 dPP, maturation of IgA transport, expression of pIgR on the SPM, and elevation of pIgR mRNA levels occur. In a previous study, cultured neonatal rat hepatocytes were shown to secrete SC into the medium when these cells were isolated from animals 20 dPP and older (18). The critical time period in both these studies corresponds well, although our data further demonstrate that low levels of SPM–pIgR protein and pIgR mRNA were detectable before 19 dPP and accounted for the low level of radiolabeled IgA transport observed. An adult pattern of secretion does not develop until the steady-state levels of pIgR mRNA and SPM–pIgR levels start to increase. Since pIgR molecules are not recycled, the development of the adult pattern of secretion is in

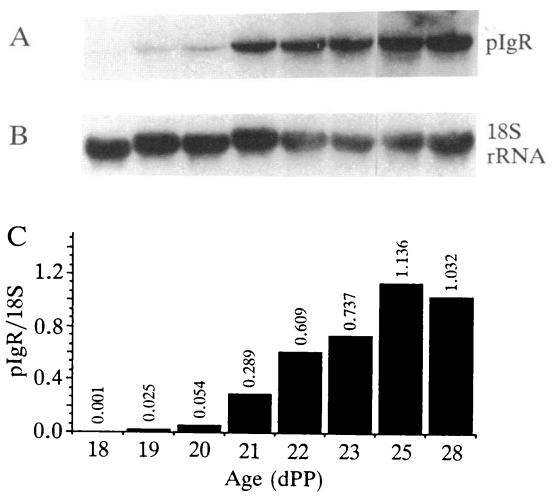

FIG. 5. Pattern of pIgR gene expression as a function of neonatal age. Total neonatal rat liver RNA (35 μg per lane) was isolated from suckling rats at various ages postpartum, electrophoresed, and blotted on to a nylon membrane (Hybond-N$^+$). (*A*) Blot hybridized to pIgR cDNA probe (GORF-1) and autoradiographed for 16 h. (*B*) Blot stripped and rehybridized to rat 18S rRNA probe and autoradiographed for 2 h. (*C*) Laser densitometry was performed and the density ratios of the pIgR band to the 18S rRNA band were calculated to control for constant loading. Numbers above the bars are the actual ratio.

all likelihood due to increased secretory capacity of the hepatocyte by virtue of a greater number of receptor molecules available to effect pIgA transcytosis. Thus, we propose that the key developmental event occurring around 21 dPP is increased transcription of the pIgR gene caused by either the appearance of a developmentally regulated transcription factor(s) or the disappearance of a developmentally regulated repressor(s). It is also conceivable that pIgR mRNA stability increases during this period.

The nature of the putative maturation factor is unknown. The data of Vaerman *et al.* (18) indicate that *in vitro* cultures of rat hepatocytes isolated from neonatal animals 20 days old or younger maintained a reduced capacity to secrete SC, which did not increase even if these cells were maintained in culture for up to 4 days. This observation suggests that the developmentally regulated stimulus for increased SC secretion is absent in cell culture conditions. These data are consistent with the possibility that the stimulus for maturation of the hepatic secretory immune system in the rat involves factors external to the hepatocyte. Cytokines and glucocorticoids have been shown to increase pIgR production in various cell types of adult tissue. Estradiol has been shown to increase SC secretion in rat uterine tissue (40) but not in cultured rat hepatocyte cultures (41, 42). Some glucocorticoids, such as dexamethasone, in a dose- and time-dependent manner, have increased SC levels in rat uterine tissue, increased rat liver SC production, and rat serum SC levels (43). Interferon-γ increases SC production and SC membrane expression in human HT-29 cells (44, 45). Many of the stimulatory effects upon SC are inhibited by RNA synthesis inhibitors (41, 42, 46). Only the effect of insulin has been studied in the rat neonatal liver and intestine. Insulin is able to increase SC production precociously in the 10-day-old neonatal liver (45, 47), but the level of insulin needed to double the level of SC production suppresses blood glucose to levels that are 25% of normal (47). Since we observe a larger than double increase in SPM–pIgR, it is possible that insulin is not the only factor capable of maintaining the elevated steady-state levels of pIgR gene expression but may be a contributing factor. Cytokines, glucocorticoids, and hormones could all be potential developmental up-regulators of pIgR expression. Weaning could be a potential influence, but its effect has not been studied systematically. Vaerman *et al.* (18) weaned all animals at 18 dPP and found the increase in expression of SC starting at 20 dPP. In our studies we did not wean any pups until 24–25 dPP yet still found all the above events increasing at 19–22 dPP.

Considerable work must be done before the mechanisms promoting this precisely regulated developmental event will be elucidated. Nevertheless, the data presented here clearly show a critical time period in which the study of regulating factors can be focused.

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