Pancreatic Islet Cells in Preobese Yellow A^{vy}/- Mice: Relation to Adult Hyperinsulinemia and Obesity (43733)

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Abstract. **Plasma insulin levels in yellow A^{vy}/- mice begin to increase before the animals are overtly obese. Are the elevated insulin levels in yellow mice primary or secondary to the subsequent obesity? Elevated blood insulin levels in young preobese mice, due to synthesis and release of insulin by increased number of β cells, would stimulate lipogenesis, resulting in excess lipid deposition and subsequent peripheral insulin resistance. Examination of this possibility was the objective of this study. The β, α, and δ cells in the pancreata of 7-, 14-, and 21-day-old male yellow A^{vy}/A and agouti A/a (BALB/c × VY)F_1 hybrid mice were counted with immunohistochemical/morphometric techniques. The insulin and glucagon concentrations in pancreata from male and female mice of the same ages and genotypes were also assayed. In the 21-day-old male mice, the mean number of β cells/pancreas was significantly greater in the yellow mice than in the agouti mice; however, insulin content and body weight were the same. This suggests that increased β cell proliferation in yellow mice precedes any detectable genotype-specific increase in pancreatic insulin content or body weight.** [P.S.E.B.M. 1994, Vol 206]

Ectopic expression of the agouti gene in mice bearing the A^{vy} (viable yellow) or A^{y} (lethal yellow) alleles at the agouti locus (Chromosome 2) results in obesity, hyperinsulinemia and diabetes, facilitation of hyperplasia and neoplasia, enhanced somatic growth, and increased synthesis of yellow pigment (phaeomelanin) by hair follicle melanocytes (1). Normally, the agouti gene is expressed only in the skin and only during the short period of time during which the subapical yellow band, characteristic of the "agouti" hair color pattern, is formed. However, in mice carrying a dominant "yellow" mutation, the 5′-flanking promoter region of the locus is altered, resulting in expression of the gene in most, if not all, tissues (1). The DNA sequence of the agouti gene suggests that the putative agouti protein probably has paracrine functions (1).

The nature of the relationship of the obesity to the other dysregulations of cellular processes is unknown. However, since prevention of yellow pigment formation does not affect the development of obesity (2), continuous phaeomelanogenesis is not a required concomitant of the obesity.

Plasma insulin levels in yellow mice began to increase at 5–6 weeks of age before the animals were overtly obese (3). Since obesity is linked to hyperinsulinemia, the question is whether elevated insulin levels in yellow mice are primary or secondary to the subsequent obesity. For example, if there were an increased number of β cells secreting insulin in young preobese mice, the presumptively elevated insulin lev-

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els would stimulate lipogenesis, resulting in excess lipid deposition with subsequent peripheral insulin resistance. As a compensatory response to the insulin resistance, further increased insulin synthesis/release would lead to severe hyperinsulinemia.

Enhancement of hyperplasia in mammary glands (4) and bladders (5) of obese yellow mice suggests that ectopic expression of the agouti gene might also favor increased proliferation of the pancreatic β-cells in young preobese yellow mice.

Before adequate morphometric technology was available to identify and count β cells, we attempted an initial assessment of the possibility that increased numbers of β cells might be present in young yellow mice prior to hyperinsulinemia (6). Improved immunohistochemical and morphometric techniques have made it possible now to identify and count the individual β, α, and δ cells in the pancreata of preweanling male yellow A^{vy}/A and agouti A/a (BALB/c × VY)F_1 hybrid mice. β cells synthesize/secrete insulin, α cells glucagon, and δ cells somatostatin. The insulin and glucagon concentrations in pancreata from male and female mice of the same ages and genotypes were also assayed.

Materials and Methods

Animals. Mottled yellow A^{vy}/A and agouti A/a mice of precisely known age were produced by date mating BALB/cStCrlfC3Hf/Nctr dams by VY/WffC3Hf/Nctr- A^{vy} sires. Litter births were recorded twice daily about 8:00 AM and 2:00 PM. Since litters are normally weaned at 21 days, the mice used in this study remained with their parents until sacrifice.

The animals were maintained in a specific-pathogen-free (SPF) barrier-sustained environment. Each breeding pair and its litter were housed in a 6.5 × 21.6 × 12.7 inch polycarbonate cage. Sterilized hardwood chips (P. J. Murphy Forest Products Corp., Rochelle Park, NJ) were used as bedding. The mice were fed sterilized NIH-31 meal (Teklad, Inc., Madison, WI) *ad libitum*. Filtered water, heated to 93.3°C and then cooled to ambient temperature, was always available. Cages, feed, and water bottles were changed weekly. Room temperature was maintained at 22° ± 2°C and relative humidity at 50% ± 5% with 15–17 changes of air/hr. Fluorescent light cycles of 12 hr on (6:00 AM to 6:00 PM) and 12 hr off (6:00 PM to 6:00 AM) were automatically regulated.

Body and pancreas weights of the 21-day-old mice used for the cell counts were not recorded due to an oversight.

Tissue Preparation and Morphometry. The animals were weighed and then sacrificed by cervical dislocation. The entire pancreas was dissected free of fat and adjacent structures and weighed. It was flattened on filter paper and fixed intact by immersion in 10% neutral buffered formalin. After fixation for 48–72 hr, the pancreatic tissues were processed routinely and embedded in Tissue Prep II (Fisher Scientific, Pittsburgh, PA). Each pancreas was serially sectioned at 5 μ.

Adjacent sections were stained with hematoxylin and eosin for general morphology of pancreas and islets, aldehyde fuchsin for β cells (7), anti-insulin antibody for β cells, antiglucagon antibody for α cells and antisomatostatin antibody for δ cells. Pancreas sections from a normal adult mouse served as positive and negative controls for each immunostain.

The antibodies used were commercial preparations obtained from Dako Corporation (Carpintera, CA) and the PAP detection system was obtained from Signet, Inc. (Cambridge, MA). The chromogen was 2,2′-diaminobenzidine (DAB) which deposits a permanent brown reaction product at the site of the target hormone thus delineating the respective cells of origin of the hormone.

The numbers of β, α, and δ cells for each pancreas were quantified, as were the total number of islets and total islet area (μ^2), using the Jandel Automated Video Analysis (JAVA) system (Jandel Scientific, Corte Madera, CA). Using the total number of cells for each cell type and the islet area, the number of cells per mm^2 of islet tissue was calculated to determine cell densities. The immunostained sections were compared within age groups for differences between genotypes.

Hormone Assays. Pancreata for insulin and glucagon assays were excised, immediately frozen in liquid N_2, and shipped to Indianapolis in dry ice.

Each pancreas was homogenized in an acid-alcohol extraction medium (85% ethanol, 1.7% concentrated hydrochloric acid). The homogenates were left standing overnight at 4°C. Subsequently, samples were diluted with Krebs-Ringer bicarbonate buffer containing 0.086% benzamidine.

Insulin content was measured with a radioimmunoassay kit from Diagnostic Products Corporation (San Diego, CA), using rat insulin as the standard.

Glucagon content was determined by a double antibody, disequilibrium radioimmunoassay. Porcine glucagon (Eli Lilly & Co., Indianapolis, IN) was used as the standard. Radioactive tracer (catalog # NEX-207) was obtained from New England Nuclear (Boston, MA). Rabbit antiserum to glucagon (lot # 23-137-2G) was obtained from Eli Lilly & Co. (Indianapolis, IN) and used at a dilution of 1:30,000. The assay buffer contained 0.05 *M* Epps (N-[2-hydroxyethyl]piperazine-N′-[3-propane sulfonic acid]), 0.01 *M* EDTA, 0.01 *M* benzamidine, 0.05% sodium azide, 0.1% Tween 20 and 0.25% bovine serum albumin. Separation of bound from free antigen was by polyethylene glycol-assisted second antibody precipitation using a goat antiserum to rabbit IgG from Cambridge Medical Diagnostics

Table I. Body and Pancreas Weights and Pancreas Weight as Proportion of Body Weight in 7-, 14-, and 21-Day-Old Yellow A^{vy}/A and Agouti A/a Male and Female (BALB/c × VY)F_1 Hybrid Mice Used for Insulin/Glucagon Assays[a]

	7 Days	14 Days	21 Days	7 Days	14 Days	21 Days
	Males			Females		
Yellow						
Body weight (g)	4.38 ± 0.07	7.27 ± 0.29	9.18 ± 0.41	4.25 ± 0.19	7.42 ± 0.26	9.10 ± 0.29
Pancreas weight (mg)	6.00 ± 0.60	17.20 ± 0.81	51.40 ± 1.52	7.45 ± 0.41	17.50 ± 0.91	46.63 ± 1.60
$\left(\frac{\text{mg pancreas}}{\text{g Body wt}}\right) \times 100$ (%)	0.14 ± 0.02	0.24 ± 0.01	0.55 ± 0.03	0.18 ± 0.01	0.24 ± 0.01	0.51 ± 0.01
n	10	10	5	11	10	8
Agouti						
Body weight (g)	4.43 ± 0.12	7.29 ± 0.26	8.75 ± 0.40	4.36 ± 0.10	7.33 ± 0.17	8.56 ± 0.38
Pancreas weight (mg)	7.00 ± 0.50	17.00 ± 1.05	46.63 ± 1.84	7.67 ± 0.61	15.90 ± 1.04	47.00 ± 1.67
$\left(\frac{\text{mg Pancreas}}{\text{g Body Wt.}}\right) \times 100$ (%)	0.16 ± 0.01	0.23 ± 0.01	0.54 ± 0.03	0.18 ± 0.02	0.22 ± 0.01	0.56 ± 0.02
n	8	10	8	12	10	9

[a] Values are given as mean ± SE.

(Billerica, MA). The detection limit of the assay was approximately 13 pg/ml.

Statistical Analyses. Significance of differences was determined by ANOVA and two-sided *t* tests.

Results

Body and Pancreas Weights. Pancreas weights in both males and females increased faster with age than did the body weights (Table I). Between 7 and 14 days mean pancreas weights in the mice used for hormone assays increased about 138%, and about 181% between 14 and 21 days. Body and pancreas weights of the male mice used for cell counts were similar to those of the males used for the hormone assays.

Islets of Langerhans. Total,[a] as well as mean,[b] islet areas did not differ significantly among any of the age or genotype groups. Due to resource limitations, islet area measurements and cell counts were performed only on male mice since the yellow males are more susceptible to development of diabetes than the yellow females. The degree of obesity and its time of development are similar in yellow males and females.

β Cells. In the 21-day-old mice, the mean number of β cells/pancreas was greater ($P < 0.001$) in yellow mice than in agouti mice (Fig. 1a). While the mean number of β cells in the 14-day-old mice, both yellow and agouti, appeared higher than in the 7-day-old mice, these differences were not statistically significant.

The mean density of β cells[c] did not differ between the 7- and 14-day-old mice, but, by 21 days of age, had increased significantly ($P < 0.05$) in the yellow mice but not in the agouti mice.

α Cells. The mean number of α cells/pancreas in both yellow and agouti mice, increased steadily from ~141 at 7 days to ~352 at 21 days of age (Fig. 1b). At 7 days of age, α cells constituted 21% of the total counted cell population. The proportion gradually increased to about 30% in the yellow and 36% in the agouti mice at 21 days of age. At all three ages, there were no statistically significant differences in density[d] between yellow and agouti mice.

δ Cells. The mean number of δ cells/pancreas in both yellow and agouti mice increased rapidly from ~3 to 7 days of age to ~80 at 14 days to ~167 at 21 days of age (Fig. 1c). Mean δ cell density[e] increased 17–20× between 7 and 14 days and doubled again by 21 days. At 7 days of age fewer than 1% of the counted cells were δ cells. This proportion increased to 8% at 14

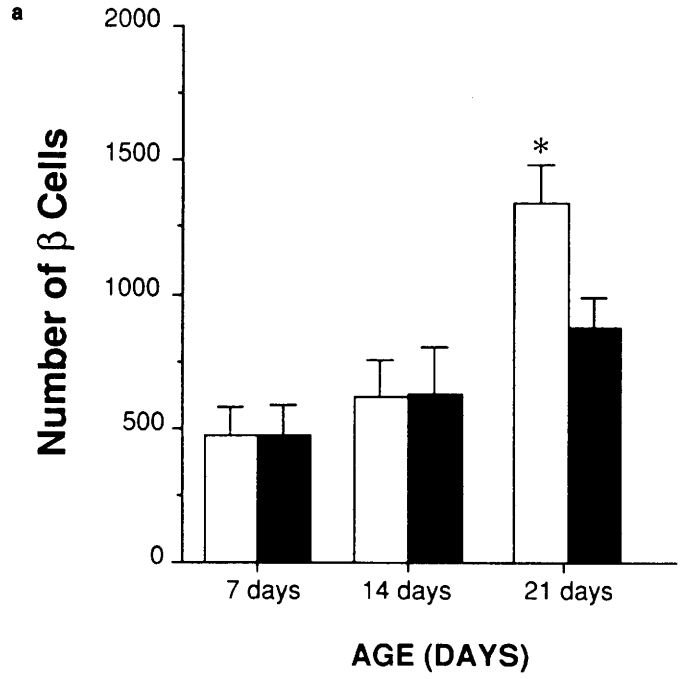
[a] Total islet areas (mean mm^2 ± SE [n]) in 7-, 14-, and 21-day-old yellow (0.207 ± 0.056 [9], 0.261 ± 0.052 [8], 0.265 ± 0.03 [8]) and agouti (0.176 ± 0.038 [9], 0.208 ± 0.079 [8], 0.228 ± 0.035 [8]) male mice.

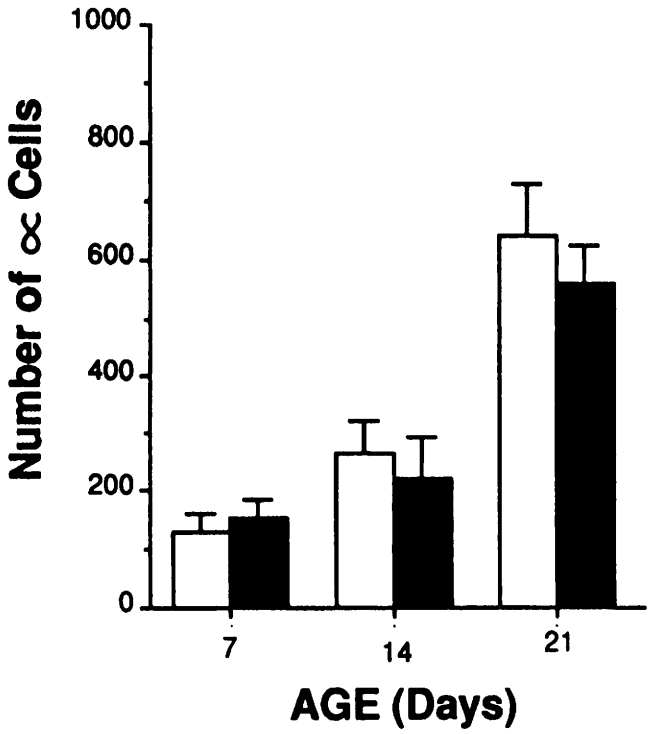
[b] Mean islet areas (mean μ^2 ± SE [n]) in 7-, 14-, and 21-day-old yellow (6224 ± 910 [9], 6405 ± 1151 [8], 7134 ± 622 [8]) and agouti (5404 ± 572 [9], 5656 ± 881 [8], 5911 ± 413 [8]) male mice.

[c] Density (mean cells/mm^2) of β cells in 7-, 14-, and 21-day-old yellow (3476 ± 290 [9], 2784 ± 255 [8], 5365 ± 672 [8]) and agouti (3576 ± 368 [9], 3036 ± 119 [8], 4028 ± 353 [8]) male mice.

[d] Density (mean cells/mm^2) of α cells in 7-, 14-, 21-day-old yellow (928 ± 79 [9], 1028 ± 131 [8], 2651 ± 364 [8]) and agouti (915 ± 74 [9], 1001 ± 94 [8], 2848 ± 538 [8]) male mice.

[e] Density (mean cells/mm^2) of δ cells in 7-, 14-, and 21-day-old yellow (17 ± 7 [9], 350 ± 35 [8], 703 ± 90 [8]) and agouti (20 ± 4 [9], 348 ± 36 [8], 820 ± 132 [8]) male mice.





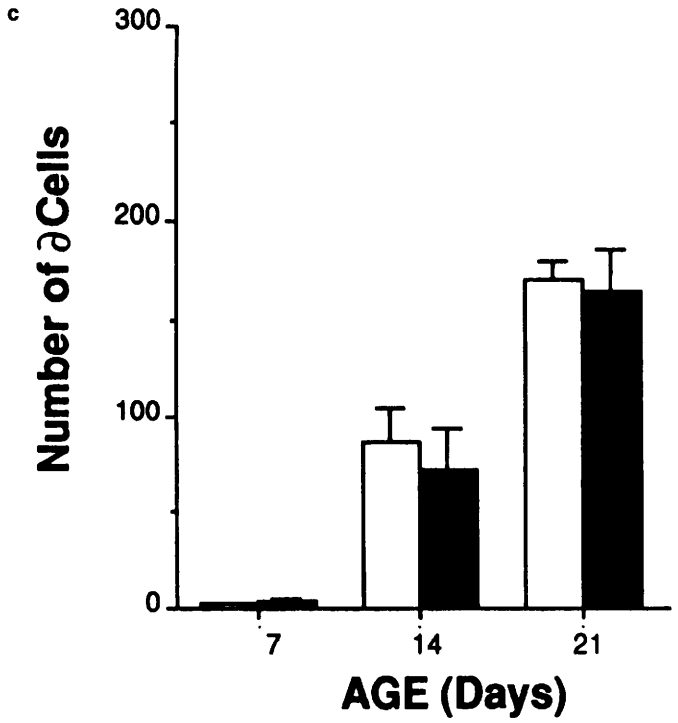


Figure 1a. Mean numbers of β cells in pancreata of yellow A^{vy}/A (open bars) and agouti A/a (closed bars) male (BALB/c × VY)F_1 hybrid mice at 7, 14, and 21 days of age. Error bars = SEM. *At 21 days the mean number of β cells/pancreas was greater ($P < 0.001$) in yellow than in agouti mice.
Figure 1b. Mean numbers of α cells in pancreata of yellow A^{vy}/A (open bars) and agouti A/a (closed bars) male (BALB/c × VY)F_1 hybrid mice at 7, 14, and 21 days of age. Error bars = SEM.
Figure 1c. Mean numbers of δ cells in pancreata of yellow A^{vy}/A (open bars) and agouti A/a (closed bars) male (BALB/c × VY)F_1 hybrid mice at 7, 14, and 21 days of age. Error bars = SEM.

days and remained essentially the same at 21 days of age. Again there were no statistically significant differences between yellow and agouti mice at any of the three ages.

Pancreatic Insulin and Glucagon. Within the 7- and 14-day-old groups there were no differences in total pancreatic insulin (Fig. 2a) or glucagon (Fig. 2b) between genotypes or sexes. However, when the data from all 7-day-old mice (male and female, yellow and agouti) were compared with the data from all 14-day-old mice, total insulin content per pancreas was higher ($P < 0.001$) at 14 than at 7 days; this is in accord with the observations of Dore *et al.* (8). In contrast, total glucagon per pancreas was lower ($P < 0.001$) at 14 days than at 7 days. Due to technical problems with the assay, the pancreatic glucagon contents for the 21-day-old mice are not available.

No changes in total insulin content per pancreas were noted between 14 and 21 days of age.

On a per mg pancreas basis, insulin concentration decreased with increasing pancreas weight between 7 and 21 days (Fig. 3a), as did the glucagon concentra-

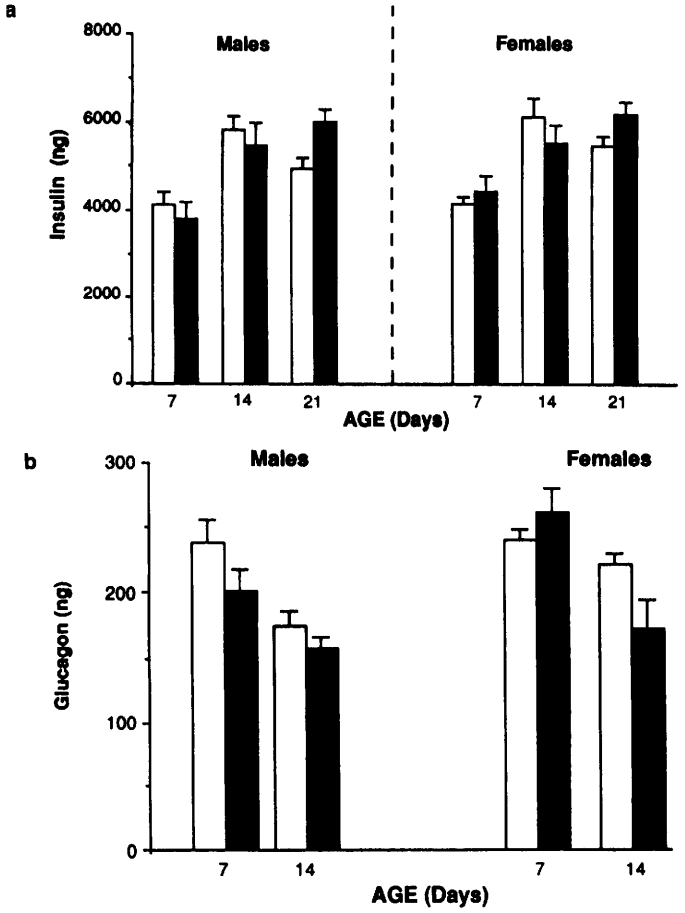


Figure 2a. Total mean insulin content (ng) of pancreata of 7-, 14-, and 21-day-old yellow A^{vy}/A (open bars) and agouti A/a (closed bars) male and female (BALB/c × VY)F_1 hybrid mice. Error bars = SEM.

Figure 2b. Total mean glucagon content (ng) of pancreata of 7-, 14-, and 21-day-old yellow A^{vy}/A (open bars) and agouti A/a (closed bars) male and female (BALB/c × VY)F_1 hybrid mice. Error bars = SEM.

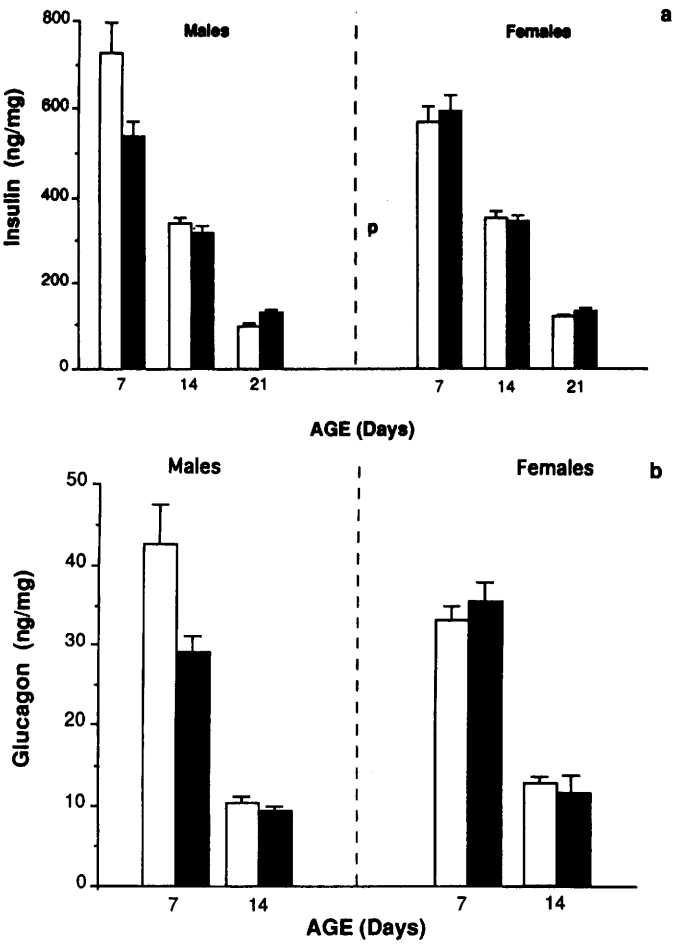


Figure 3a. Mean insulin content per mg pancreas of 7-, 14-, and 21-day-old yellow A^{vy}/A (open bars) and agouti A/a (closed bars) male and female (BALB/c × VY)F_1 hybrid mice. Error bars = SEM.

Figure 3b. Mean glucagon content per mg pancreas of 7-, 14-, and 21-day-old yellow A^{vy}/A (open bars) and agouti A/a (closed bars) male and female (BALB/c × VY)F_1 hybrid mice. Error bars = SEM.

tion between 7 and 14 days (Fig. 3b). This suggests that hormone content is independent of growth of the pancreas.

Discussion and Conclusions

In an earlier study (6), the size distribution of islets of Langerhans in yellow and agouti (BALB/c × VY)F_1 hybrid male mice was compared at daily intervals between 21 and 28 days of age and at 31, 35, 42, and 63 days. The data indicated that the mean proportion of "small" islets was lower ($P < 0.05$) in the yellow mice, i.e., the average islet size was somewhat greater in the yellow than in the agouti mice islets (Table II).

Plasma insulin concentrations (assayed by L. G. Frigeri) in male yellow and agouti (BALB/c × VY)F_1 mice started to diverge on Day 23 increasing to Day 25. An unexplained decrease in the insulin level then was observed in the yellow mice until day 27. At this time, the plasma insulin levels of yellow and agouti mice started to diverge again and continued to do so to the end of the study at Day 63 (Fig. 4).

Plasma glucose concentrations (Fig. 4) were also measured on mice at these ages. Glucose levels were higher in yellow than in agouti mice beginning at Day 24 and remained so to the end of the study.

The present study asked whether a larger number of pancreatic β cells, as potential sites of increased insulin synthesis and release, might differentiate very young $A^{vy}/-$ mice from non-A^{vy} mice. The data indicate that there were significantly more β cells in the pancreata of yellow mice than in those of agouti mice at 21 days of age. This suggests that small proliferative rate differences between β cells in yellow and agouti mice may begin to occur between 14 and 21 days of age. The lack of a difference in pancreatic insulin content between yellow and agouti mice at 21 days may be attributable to a lag in insulin synthesis by the proliferating β-cells. In the earlier study, plasma insulin levels

Table II. Proportion (%) of "Small" Islets of Langerhans in Male (BALB/c × VY)F_1 Hybrid Mice from 21–63 Days of Age[a]

Age (days)	Yellow (A^{vy}/A)	Agouti (A/a)
21	65.8	71.3
22	66.6	72.8
23	65.0	69.1
24	69.8	73.3
25	66.7	68.2
26	65.6	61.9
27	63.3	68.9
28	63.3	68.9
Mean	66.2[b]	69.1
SE	0.8	0.8
31	—	69.5[c]
35	60.4	63.3
42	57.8	61.6[d]
63	51.9	66.7

[a] Values are given as mean ± SE (n = 12, except as noted). Methodological details are provided in ref. 6 from which these data are reproduced with permission.
[b] $P < 0.05$ (yellow < agouti)
[c] $n = 6$
[d] $n = 7$

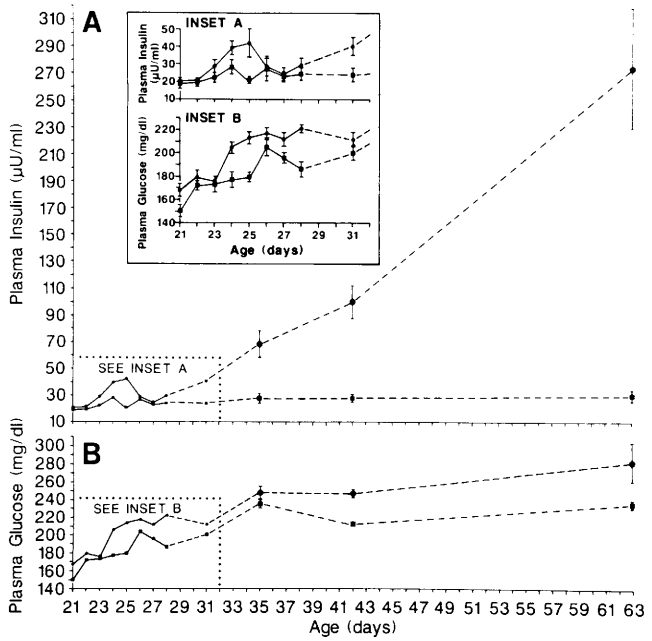


Figure 4. Plasma insulin and glucose concentrations in yellow A^{vy}/A (-■-) and agouti A/a (-●-) male (BALB/c × VY)F_1 hybrid mice between 21 and 63 days of age. Error bars = SEM. (Reproduced from Ref. 6 with permission.)

between yellow and agouti mice began to diverge at 23 days of age.

At the ages examined in the present study, the relation of pancreas to body weight appears to be strain-specific since Dore *et al.* (8) observed rather different relationships in the inbred SWR mouse strain. At 7 days, our body weights were about the same as their 10-day weights, while our pancreas weights were within the range of their 5-day weights. At 14 days, our body weights were about 50% greater than their 15-day-old weights, while our pancreas weights appeared to cluster around their 15-day weights. At 21 days, our body weights were 40% greater than their 20-day weights, while our pancreas weights were about 25% greater than theirs.

In a recent investigation (9), plasma insulin levels differed between female yellow and agouti (BALB/c × VY)F_1 hybrid mice at the start of the study at 25–32 days of age and continued to differ significantly to the end of the study at 179–186 days of age.

In earlier work (3), increased body weight of yellow A^{vy}/A (BALB/c × VY)F_1 hybrid mice, relative to their nonyellow sibs, began to be detectable about 5–6 weeks of age; but the animals did not start to become overtly obese until about 8 weeks of age. Plasma insulin levels in the male yellow mice began to increase, in comparison with the agouti A/a mice, at 5–6 weeks of age.

While neither the identity nor the site of action of the primary initiating lesion leading to obesity is known, hypothalamic dysregulation resulting in increased food consumption and increased efficiency of feed utilization is considered a possibility (10). A perturbed hypothalamus-pituitary-adrenal axis causing an increase in the parasympathetic tone and a decrease in the sympathetic tone would result in insulin oversecretion (11) which is present in both genetic obesity and in obesity induced by hypothalamic lesions (12). Rohner-Jeanrenaud *et al.* (13, 14) found that 17-day-old preobese *fa/fa* rats, when challenged with an intravenous glucose load, oversecreted insulin when compared with their normal *Fa/-* sibs. They observed that this oversecretion could be inhibited by atropine, indicating that it is mediated by the efferent vagus nerve. Jeanrenaud (12) stated ". . . insulin oversecretion is an early abnormality, that can therefore be causal in the aetiology of obesity." The recent reports that agouti RNA is present in the brains of yellow $A^{y}/-$ (15, 16) and $A^{vy}/-$ mice (1) implies the presence of agouti protein in the brains of yellow mice, but not in those of agouti $A/-$ mice. Therefore, once this protein becomes available for experimental use, its possible role in such neuroendocrine dysregulation might be explored.

The results of the present study suggest that increased β cell proliferation in yellow mice occurs between 14 and 21 days of age, preceding any detectable genotype-specific increase in pancreatic insulin content or body weight. The identities and sequences of developmental pathways and processes, affected by the ectopic presence of the agouti protein, which result in hyperinsulinemia and obesity in adult yellow mice remain to be elucidated.

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The plasma insulin assay data in Figure 4 were generated by Dr. Luciano G. Frigeri while at the Whittier Institute for Diabetes and Endocrinology, La Jolla, CA.

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