Overexpression of arginase I in enterocytes of transgenic mice elicits a selective arginine deficiency and affects skin, muscle, and lymphoid development[1–3]

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ABSTRACT

Background: Arginine is required for the detoxification of ammonia and the synthesis of proteins, nitric oxide, agmatine, creatine, and polyamines, and it may promote lymphocyte function. In suckling mammals, arginine is synthesized in the enterocytes of the small intestine, but this capacity is lost after weaning.

Objective: We investigated the significance of intestinal arginine production for neonatal development in a murine model of chronic arginine deficiency.

Design: Two lines of transgenic mice that express different levels of arginase I in their enterocytes were analyzed.

Results: Both lines suffer from a selective but quantitatively different reduction in circulating arginine concentration. The degree of arginine deficiency correlated with the degree of retardation of hair and muscle growth and with the development of the lymphoid tissue, in particular Peyer's patches. Expression of arginase in all enterocytes was necessary to elicit this phenotype. Phenotypic abnormalities were reversed by daily injections of arginine but not of creatine. The expression level of the very arginine-rich skin protein trichohyalin was not affected in transgenic mice. Finally, nitric oxide synthase–deficient mice did not show any of the features of arginine deficiency.

Conclusions: Enterocytes are important for maintaining arginine homeostasis in neonatal mice. Graded arginine deficiency causes graded impairment of skin, muscle, and lymphoid development. The effects of arginine deficiency are not mediated by impaired synthesis of creatine or by incomplete charging of arginyl–transfer RNA. *Am J Clin Nutr* 2002;76:128–40.

KEY WORDS Arginine, arginase I, Peyer's patch, myocyte, hair, immunonutrition, enterocytes, transgenic mice

INTRODUCTION

Arginine is a precursor for the synthesis of proteins, nitric oxide, agmatine, creatine, urea, and polyamines and is an intermediate in the detoxification of ammonia to urea. Of these, protein, creatine, and urea synthesis are quantitatively most important. Endogenous arginine biosynthesis in adult mammals occurs mainly in the proximal convoluted tubules of the kidney (1) from citrulline (2) that, in turn, is formed in the enterocytes of the small intestine from glutamine and proline (3–5). The endogenous biosynthesis of arginine is low in strict carnivores such as cats and ferrets, intermediate in humans, and relatively high in rapidly and continuously growing animals such as rats and pigs (6). In adult humans, the endogenous biosynthetic capacity for arginine meets the daily requirement under steady state conditions (7), but a dietary source of arginine may become necessary when demand increases under anabolic or catabolic conditions (7). For this reason, arginine is now considered a conditionally essential amino acid.

In rapidly growing suckling rats, humans, and pigs, the dietary supply of arginine via the milk does not meet the requirement for arginine accumulation in proteins (8, 9), implying that endogenous arginine biosynthesis must be important during this period. Evidence is accumulating that the intestine rather than the kidney plays a major role in arginine biosynthesis in the suckling period. In concert, the enterocytes of the small intestine express the enzymes required for arginine production from glutamine and proline (10–12) and do not express arginase (EC 3.5.3.1) (12). The intestine appears to play a crucial role in arginine biosynthesis not only in rapidly growing neonates such as

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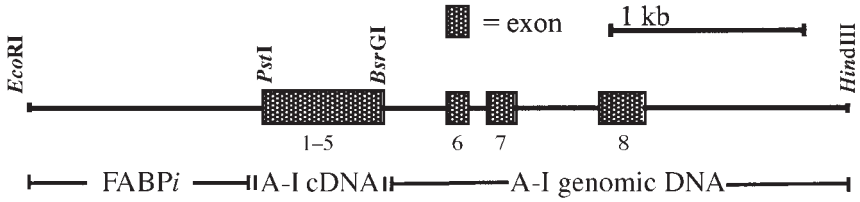


FIGURE 1. Chimeric fatty acid–binding protein (FABP*i*)–arginase DNA construct used to generate F/A transgenic mice. The 4.3-kilobase (kb) DNA fragment consists of the −1178– to +28–base pair (bp) *Eco*RI-*Bam*HI rat intestinal FABP*i* promoter-enhancer fragment (21), the 520-bp *Pst*I-*Bsr*GI rat arginase I (A-I) complementary DNA fragment containing exons 1–5, and the 2400-bp *Bsr*GI-*Hin*dIII rat A-I genomic DNA fragment containing exons 6–8 (22).

rodents and pigs but also in neonatal humans, because destruction of the enterocytes in necrotizing enterocolitis also results in a selective decrease in circulating arginine (13). After weaning, argininosuccinate synthetase (ASS; EC 6.3.4.5) and argininosuccinate lyase (EC 4.3.2.1), the enzymes that synthesize arginine from citrulline, disappear from the enterocytes in mice and rats, concurrent with the appearance of endogenous arginase (12), so that only the capacity to synthesize citrulline remains.

Arginine deficiency is known to cause a life-threatening hyperammonemia in preterm infants (14). Furthermore, arginine deficiency in growing animals is usually associated with growth retardation (7, 15, 16). However, it is not known whether arginine deficiency also affects developmental processes other than growth and if so, what the quantitative relation between arginine deficiency and these sequelae is. Furthermore, the regulatory mechanisms underlying the development of these sequelae in growing mammals must be elucidated to better understand the role of arginine metabolism in normal growth, vessel wall biology (17, 18), and immunologic responsiveness (19, 20). We therefore developed a transgenic mouse model that suffers from a selective decrease in circulating and tissue arginine as a result of overexpression of arginase I (A-I) in the enterocytes of the small intestine. The phenotypic deficits in the growth and development of hair, muscle, and the immune system in this transgenic model highlight the importance of the small intestine for arginine metabolism in the suckling period.

MATERIALS AND METHODS

Mice

Mice were kept under environmentally controlled conditions (light on from 800 to 2000; water and standard, unpurified rodent diet ad libitum; 20–22°C; 55% humidity). Litters discovered in the morning were assigned neonatal day 0 (ND0). Pups were weaned at 3 wk of age. Mice of the FVB strain were used for generation of transgenics. Mice deficient in sparse-fur-abnormal-skin-and-hair (*Spf-ash*), nitric oxide synthase 1 (NOS1; EC 1.14.13.39), NOS2, and NOS3 had a C57/Bl6 background and were purchased from the Jackson Laboratory, Bar Harbor, ME. Animal experiments were performed in accordance with the guidelines of the local animal research committee, which follows the guidelines of the Academic Medical Center of the University of Amsterdam.

Nest size was adjusted to 7 pups. They received subcutaneous injections of 5 mmol/kg of 150 mmol/L arginine-HCl or creatine-monohydrate in the back twice daily (0900 and 1600). Control animals were injected with the same volume of 0.9% NaCl. The mice were weighed daily at 1500. Each weight curve is based on repeated measurements of 10–16 animals. Male and female weight curves did not differ significantly.

Targeting arginase I expression to the enterocytes

The 4.3-kb construct used for generation of the transgenic animals (**Figure 1**) is a chimera of the −1178- to +28–base pair (bp) fragment of the rat intestinal fatty acid–binding protein (*FABPi*) promoter-enhancer element (23), the 520-bp rat *A-I* complementary DNA fragment, containing exons 1–4 (24), and the 2400-bp rat *A-I* genomic DNA fragment, containing exons 5–8 (22). Polymerase chain reaction of tail-tip DNA was carried out at 56°C with the use of 5′-AAATGCCTACATGCTGTAGTCGG-3′, complementary to nucleotide −218 to −196 of the *FABPi* promoter-enhancer DNA as upstream primer, and 5′-CCAATTGCCATACTGTGGTCTCC-3′, complementary to nucleotide +320 to +342 of the *A-I* complementary DNA as downstream primer. Transgene copy number was determined by Southern blotting and was 5–6 in both lines studied. Putative homozygous mice were crossed with wild-type mice, and a sample of 15 pups was analyzed by polymerase chain reaction screening to confirm the genotype. Homozygous mice of line F/A-1 and hemizygous mice of line F/A-2 were used for breeding. Homozygous F/A-2 mice were identified by phenotype and weight (**Figure 2**).

Determination of amino acid, creatine, and ammonia concentration

Pups were separated from their mothers and kept at 37°C for 1 h before being killed. After decapitation, blood was collected into heparin-containing tubes and centrifuged at 2000 × *g* for 5 min at 4°C. Fifty microliters plasma was added to 4 mg lyophilized sulfosalicylic acid, centrifuged at 16000 × *g* for 5 min at 4°C, and stored at −70°C. Tissue samples were collected; flushed in ice-cold phosphate-buffered saline (PBS) at pH 7.4, rapidly frozen in liquid nitrogen; and stored at −70°C. Approximately 80 mg tissue was added to 400 μL of a 5% sulfosalicylic acid solution containing 300 mg glass beads with a diameter of 1 mm. The tissue was homogenized for 30 s in a Mini Bead-Beater (BioSpec Products, Bartesville, OK) and centrifuged at 16000 × *g* for 5 min at 4°C. Jejunum and plasma amino acids were determined by fully automated HPLC as described (25). Proline and cysteine are not detected with this method. Norvaline was used as an internal standard. Cadaverine was determined to assess residual bacterial content in the intestinal lumen. For analysis of guanidino compounds, plasma was deproteinized with an equal volume of 20% trichloroacetic acid. Guanidino compounds were separated on a cation-exchange column and detected as described (26). Urea nitrogen was determined with diacetylmonoxime, as described (27). Blood

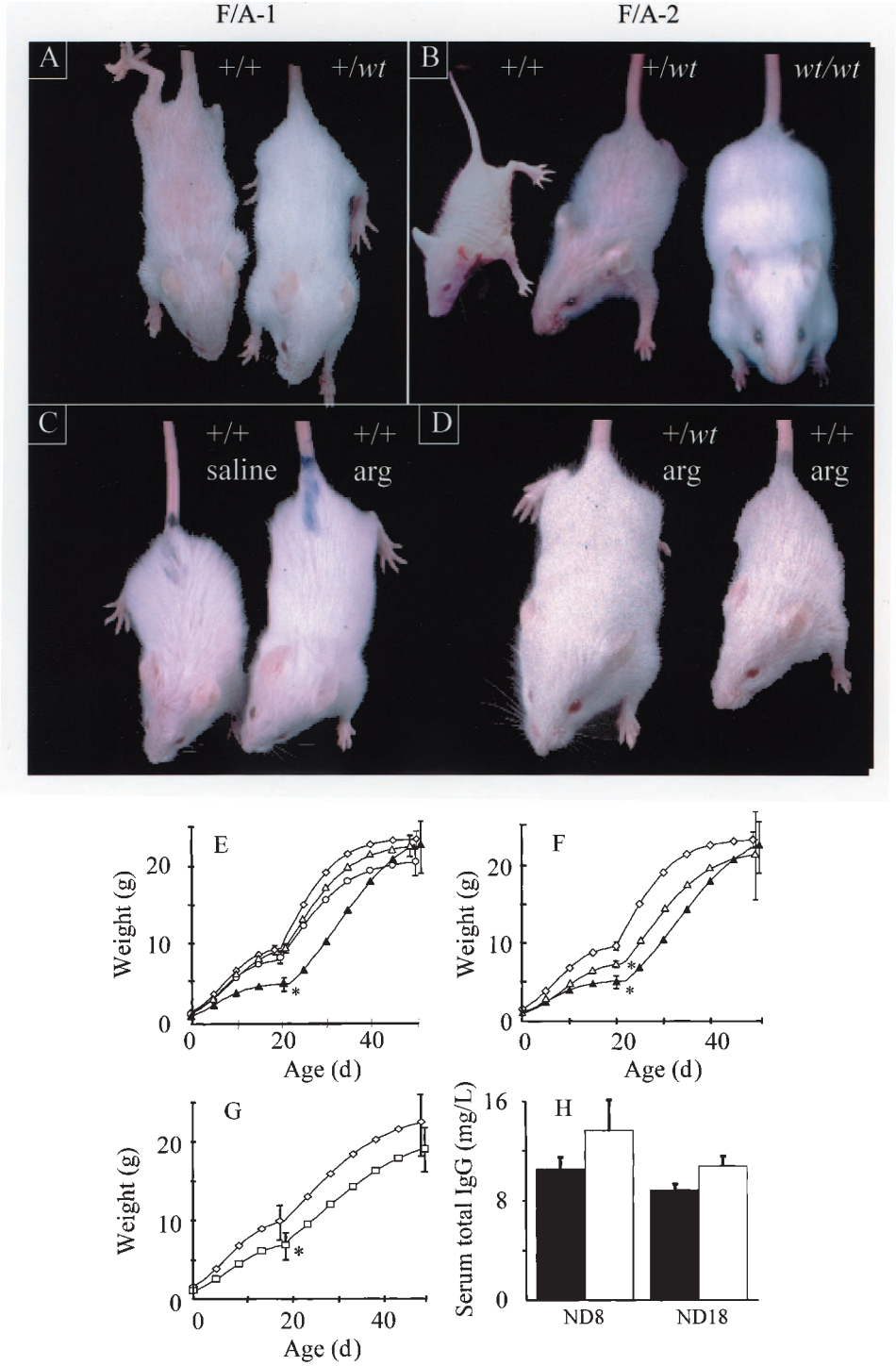


FIGURE 2. Appearance and body weight of F/A-1 and F/A-2 transgenic mice. Homozygous (+/+) and hemizygous (+/*wt*) F/A-1 mice are shown in panel A, and homozygous and hemizygous F/A-2 and wild-type (*wt/wt*) mice in panel B, all at neonatal day 20 (ND20). Note the difference in fur development and body size, in particular the similarity of F/A-$1^{+/+}$ and F/A-$2^{+/wt}$ mice and of F/A-$1^{+/wt}$ and wild-type mice. C: Injection of arginine (right mouse) but not saline (0.9% NaCl; left mouse) restores fur development in F/A-$1^{+/+}$ mice. D: Injection of arginine causes recovery of F/A-$2^{+/wt}$ mice (left mouse) but only partial recovery of F/A-$2^{+/+}$ mice (right mouse). E: Weight gain of F/A transgenics: wild type (◇), F/A-$1^{+/+}$ (○), F/A-$2^{+/wt}$ (△), F/A-$2^{+/+}$ (▲). F/A-$2^{+/+}$ mice have a significantly ($P < 0.01$) lower body weight at weaning but fully recover as adults. F: Weights of F/A-$2^{+/+}$ mice injected with saline (▲) and arginine (△), compared with wild-type animals (◇). At ND21, arginine-injected F/A-$2^{+/+}$ mice had significantly greater body weight than that of saline-injected F/A-$2^{+/+}$ mice but did not reach wild-type weight (for both, $P < 0.01$). G: Weight curve of ornithine transcarbamoylase–deficient sparse-fur-abnormal-skin-and-hair (*spf-ash*) mice (□) and wild-type C57/Bl6 mice (◇). At ND21, the body weight of *spf-ash* mutant mice is significantly ($P < 0.01$) lower than that of wild-type mice. Error bars depict a 99% CI of fitted growth curves. H: Serum total immunoglobulin G (IgG) concentrations in wild-type and F/A-$2^{+/+}$ mice at ND8 and ND18. ■, wild type; □, F/A-$2^{+/+}$. Serum total immunoglobulin concentrations are not different in the 2 genotypes. For each measurement, serum of 3 mice was pooled. Values are ±SEM and are based on 3 independent analyses.

ammonia concentration was determined with an ammonia test kit (Menarini Diagnostics, Florence, Italy), according to the manufacturer's instructions.

Histology

For in situ hybridization, tissues were fixed in 4% formaldehyde in PBS, pH 7.4, for 4 h at 4°C and overnight at room temperature. In situ hybridizations were carried out as described (28). The A-I probe was the 768-bp *Pst*I-*Stu*I fragment of clone pARGr-2 (24). For immunohistochemistry, tissues were fixed in methanol:acetone:water (2:2:1; by vol) for 4 h at 4°C and overnight at room temperature, embedded in paraplast, and sectioned at 7 μm. After deparaffination, endogenous peroxidase activity was eliminated by treatment with 3% hydrogen peroxide in 50% methanol. Nonspecific binding was blocked by incubation for 30 min in TENG-T (10 mmol tris/L, 5 mmol EDTA/L, 150 mmol NaCl/L, 0.25% gelatin, 0.05% Tween-20; pH 8.0). Serial sections of intestines were incubated overnight with an appropriate dilution of a rabbit antiserum against ASS (29, 30), A-I (31), carbamoylphosphate synthetase (EC 6.3.4.16) (32), and sucrase-isomaltase (SI; EC 3.2.1.48) (33). Rabbit anti–rat A-II antiserum was raised against amino acids 337–350 of rat A-II (34) coupled to keyhole limpet hemocyanin. The indirect unconjugated peroxidase–antiperoxidase technique (35) was used to visualize binding of the primary antibodies. Sections of skin were stained with a polyclonal antibody against trichohyalin (36). For whole-mount immunostaining, small intestines of ND6 mice were fixed for 16 h in methanol-dimethylsulfoxide (4:1) at 4°C with gentle agitation and incubated with a monoclonal fluorescein isothiocyanate–conjugated antibody against murine vascular cell adhesion molecule 1 (VCAM-1; 1 μg/mL in PBS; PharMingen, San Diego). After washing, tissues were incubated for 2 h at 37°C with horseradish peroxidase–conjugated anti–fluorescein isothiocyanate antibodies (POD converter kit; Boehringer Mannheim, Mannheim, Germany) and stained with diaminobenzidine and hydrogen peroxide in the presence of nickel chloride.

Western blotting

Jejunal tissue and shaven skin were homogenized in 20 mmol phenylmethylsulfonyl fluoride/L, 1 mmol EDTA/L, and 1 mmol DL-dithiothreitol/L. Twenty micrograms of protein (bicinchoninic acid assay; Pierce, Rockstone, IL) was separated on 10% sodium dodecyl sulfate polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). Membranes were blocked by overnight incubation in 10% nonfat milk in PBS and subsequently incubated with primary antibodies. Goat anti–rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (EC 3.1.3.1) was used as secondary antibody.

Quantification of serum immunoglobulin concentration

Serum was collected from 8- and 18-d-old wild-type and homozygous F/A-2 littermates by decapitation. Total IgG concentration was quantified with the use of an enzyme-linked immunosorbent assay according to the manufacturer's instructions (Southern Biotechnology Associates, Birmingham, AL).

Arginase activity assay

Intestinal tissue was homogenized in 40 volumes of 30 mmol sucrose/L, 5 mmol $MnCl_2$/L, 30 mmol imidazole buffer/L (pH 7.5), and 0.05% Triton X-100. This homogenate was sonicated on ice for 2 min, activated at 55°C for 5 min, and centrifuged at 10000 × *g* for 10 min at 4°C. The assay mixture (200 μL) contained enzyme extract, 25 mmol (guanidino-^{14}C)-L-arginine/L (pH 9.5, 15 mCi/mol; Dupont NEN, Boston), and 75 mmol glycine/L (pH 9.5). After 10 min at 37°C, the reaction was stopped by loading the assay mixture on a cation-exchange column (Bio-Rad, Richmond, CA). Urea was eluted with 1 mL tris-EDTA buffer, pH 8.5, and counted. Protein content was determined with the bicinchoninic acid assay.

Statistics

Weight data were fitted to a logistic growth curve ($weight_t = weight_{max}/(1+e^{-b(t-t0)})$). Fitting the curve in 2 stanzas (preweaning and postweaning) resulted in a significantly better fit (*F* test on reduction of residual variation). To compare the resulting biphasic weight curves between genotypes, treatments, or both, a 99% CI was constructed both at 3 wk (the age of weaning) and at 7 wk (adolescence). Biochemical data on arginase, amino acids, immunoglobulins, and guanidino compounds were tested with a repeated-measure analysis of variance (ANOVA) for each compound group. Because of significant interactions between compound, genotype, and age, a two-way ANOVA (arginase, amino acids, and immunoglobulins; factors age and genotype) or a one-way ANOVA (guanidino compounds; factor genotype) was carried out for each compound. In case of a significant effect of genotype or genotype-age interaction in these ANOVAs, Dunnett's multiple comparison test between genotypes was applied. Results were considered significantly different at $P < 0.01$. Counts on Peyer's patches (PPs) were tested with a Poisson distribution. SPSS, version 10.1 (SPSS Inc, Chicago) was used for the statistical analyses.

RESULTS

Intestinal arginase expression is associated with retarded body growth and fur development

Six founder mice were bred and analyzed. Two lines, designated F/A-1 and F/A-2, were clearly distinguishable from age-matched wild-type animals in that the appearance of a normal fur coat was delayed until after weaning in homozygous F/A-1 and all F/A-2 mice (Figure 2, A and B). The animals were no longer distinguishable from wild-type animals by 6–7 wk. Lines F/A-3 to -6 were indistinguishable from wild-type animals. Histologic analysis of homozygous F/A-2 skin at weaning revealed a severe distortion of hair follicles (**Figure 3**, A and D). Many hair bulbs were too high in the subcutaneous tissue, contained keratohyalin grains, and did not develop normally. Prominent keratin plugs clogged the hair follicles. In general, the appearance was that of a delayed maturation of hair. However, the onset of sebaceous gland development appeared normal. The presence of hyperkeratosis and a prominent stratum granulosum in the epidermis also points to delayed maturation. The hair follicle protein trichohyalin is extremely rich in arginine residues (36) and hence is a potential target of arginine deficiency. However, immunohistochemical staining of skin sections (Figure 3, B and E) and Western blots of skin homogenates (Figure 3, C and F) did not reveal any differences in trichohyalin content, though we did observe an absence of trichohyalin from the epidermis.

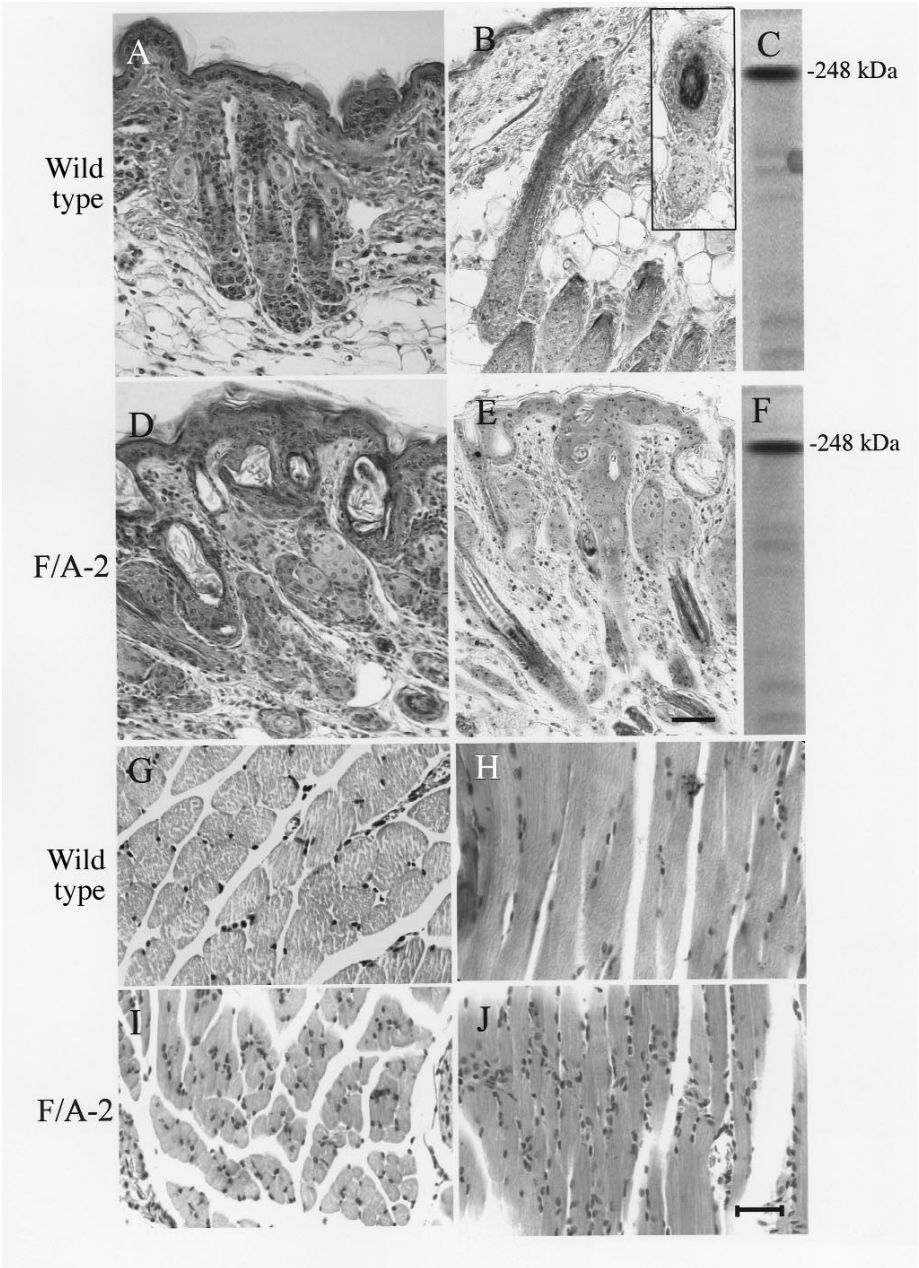


FIGURE 3. Skin and muscle development in wild-type and F/A-2$^{+/+}$ mice. A, B, D, and E: Sections of dorsal skin stained with hematoxylin and azophlochsin (A and D) or for the presence of trichohyalin (B and E) in wild-type (A and B) and F/A-2$^{+/+}$ (D and E) mice. Note the disturbed appearance of hair follicles in transgenic mice but normal staining pattern of trichohyalin in the inner root sheath in transgenic animals. C and F: Western blot of skin extract stained for the presence of trichohyalin. Note similar concentrations in both extracts. G, H, I, and J: Hematoxylin- and azophlochsin-stained sections of gastrocnemius muscle of wild-type (G and H) and F/A-2$^{+/+}$ (I and J) mice are shown at neonatal day 20. Note the regular myofiber pattern, with peripherally located nuclei, in both genotypes but also the 3-fold decrease in fiber diameter and the pronounced increase in number of peripheral nuclei in F/A-2$^{+/+}$ mice. Bar: 100 μm.

The mice showed a biphasic growth curve in the first 8 wk of life, with the most-rapid growth occurring immediately after birth and after weaning (Figure 2E). The growth rate of homozygous F/A-1 and hemizygous F/A-2 mice was normal, but homozygous F/A-2 animals exhibited a pronounced retardation in body growth before weaning, which became discernible at 5 d after birth and which amounted to a 2-fold reduction in body weight at weaning. Because muscle is an important component of body weight, we analyzed the gastrocnemius muscle of 3-wk-old homozygous F/A-2 mice histologically (Figure 3, G, H, I, and J). The diameter of the myofibers was approximately one-third that of wild types, with many nuclei located in the periphery of the fibers. No dystrophic features were observed, which suggests active myogenesis.

The fur of the F/A transgenics resembles that of the ornithine transcarbamoylase (OTC; EC 2.1.3.3)–deficient *spf-ash* mutant mice (37). The growth rate of suckling *spf-ash* mice is also retarded significantly but to a lesser extent than in F/A-2 mice (Figure 2G).

Suckling homozygous F/A-2 mice appeared to have normal feeding behavior and intestinal function. The absence of malnutrition

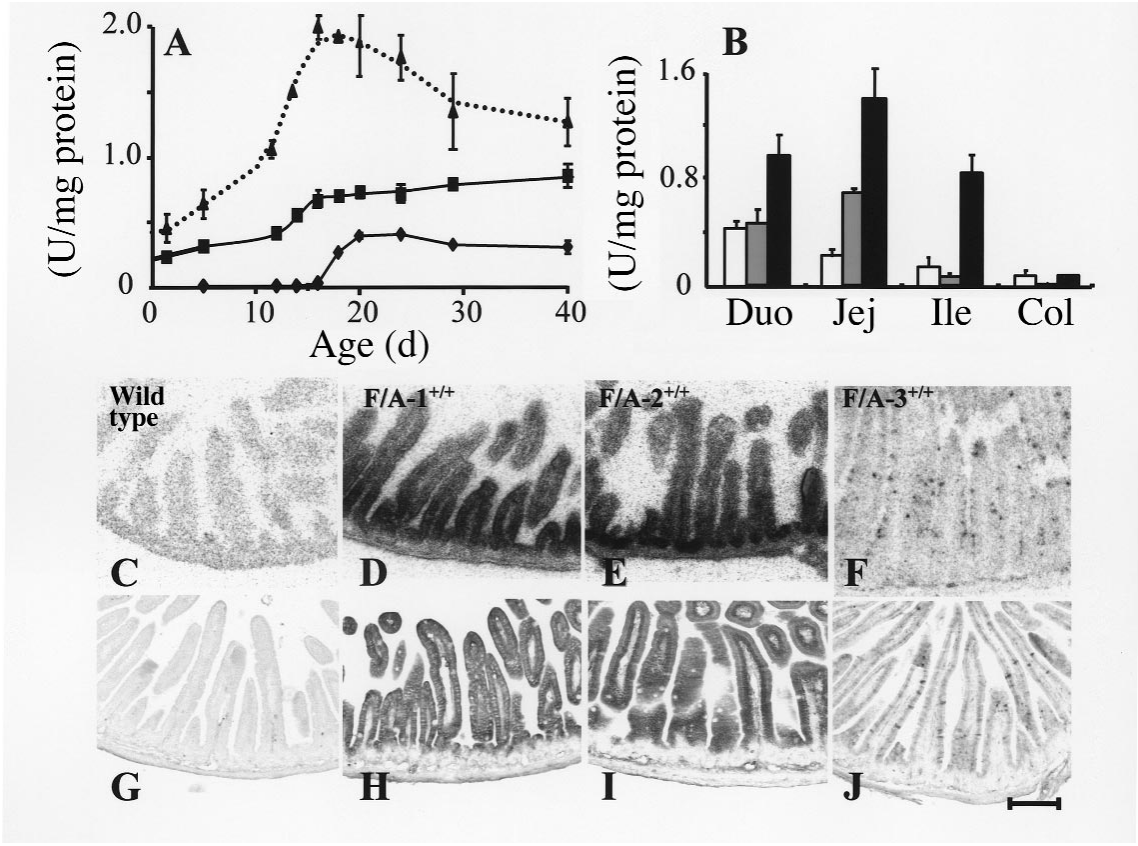


FIGURE 4. A: Arginase activity in jejunal homogenates of wild-type and transgenic mice: F/A-$2^{+/+}$ (▲), F/A-$1^{+/+}$ (■), wild-type (◆). B: Distribution of arginase activity along the proximal-distal axis of the intestine in wild-type (□), F/A-$1^{+/+}$ (■), and F/A-$2^{+/+}$ (■) mice at neonatal day 20 (ND20). The highest transgenic arginase activity is found in the jejunum (Jej), whereas endogenous arginase expression in wild-type mice is highest in the duodenum (Duo). Ile, ileum; Col, colon. Distribution of arginase I (A-I) mRNA (C, D, E, and F) and of A-I protein (G, H, I, and J) in jejunal enterocytes of wild-type (C and G), F/A-$1^{+/+}$ (D and H), F/A-$2^{+/+}$ (E and I), and F/A-$3^{+/+}$ (F and J) mice at ND8. Note that lines F/A-1 and -2 express A-I in all enterocytes, whereas F/A-3 expresses A-I in ≈5% of the enterocytes. Bar: 75 μm.

was deduced from the presence of normally filled stomachs and a normal serum concentration of immunoglobulins (Figure 2H), which are of maternal origin and have to be transported across the intestinal epithelium (38). Furthermore, the transgenic mice were not feeble but instead were hyperactive (WJ de Jonge, unpublished observation, 2001).

Intestinal arginase expression selectively reduces arginine concentration

We confirmed that carbamoylphosphate synthetase and OTC are expressed in all enterocytes of suckling mice and that ASS and argininosuccinate lyase are only expressed in enterocytes on the distal half of the villi, as we previously reported for the suckling rat intestine (12). As in suckling rats, endogenous arginase activity in the intestine did not become detectable until the third postnatal week in wild-type mice (**Figure 4**A). Arginase activity in homozygous F/A-1 mice amounted to 0.2 U/mg protein shortly after birth and rose to 0.7–0.8 U/mg protein in the second postnatal week. Arginase activity in line F/A-2 followed a similar pattern but at a 2–3-fold higher rate. The parallel rise in arginase activity in wild-type and transgenic mice at ND16 suggests that the expression of transgenic, FABP*i*-driven A-I did not interfere with the expression of endogenous A-II. Hence, the accelerated rise in enzyme activity in F/A-1 and -2 in the second postnatal week was at least partly due to the rise in endogenous arginase. At ND20, endogenous arginase activity was highest in the duodenum, whereas FABP*i*-driven A-I activity was highest in the jejunum and decreased toward the duodenum and the colon distally (Figure 4B). Hemizygous mice expressed arginase at a level intermediate to that of wild-type and homozygous mice (Figure 4A and **Table 1**).

The sparse-fur phenotype of F/A-1 and F/A-2 mice was associated with A-I expression in all enterocytes, except those in the crypts (Figure 4, D, E, H, and I). Hence, transgenic A-I colocalized with ASS and argininosuccinate lyase. Transgenic A-I protein or mRNA was not expressed in other tissues, such as the kidney, in these lines. The intestinal arginase activities in hemizygous F/A-3 to -6 mice, which do not have a phenotype, are shown in Table 1. Arginase activity in line F/A-3 exceeded that present in F/A-1 mice but, in contrast with line F/A-1, A-I in F/A-3 was expressed in only 5% of the enterocytes (Figure 4, F and J). These data show that, in addition to a high level of A-I activity, a homogeneous expression of A-I is necessary for the F/A phenotype to develop.

In suckling wild-type mice, the concentration of arginine was 200–250 μmol/L in plasma and 350–400 μmol/kg wet weight in jejunal tissues (**Figure 5**A). In the homozygous suckling F/A-1 and -2 mice, plasma and jejunal arginine concentrations were reduced to, respectively, 90–110 and ≈75 μmol/L and 110–140 and ≈80 μmol/kg. Arginine concentrations in F/A-1 mice

TABLE 1
Intestinal arginase activity in wild-type and F/A transgenic mice[1]

Line	Arginase activity
	U/mg protein
Wild type	0.007 ± 0.003
F/A-1	0.208 ± 0.003[2]
F/A-2	0.450 ± 0.006[2]
F/A-3	0.289 ± 0.031[2]
F/A-4	0.169 ± 0.026[2]
F/A-5	0.084 ± 0.017[2]
F/A-6	0.221 ± 0.047[2]

[1] $\bar{x}$ ± SEM; $n = 6$. Arginase activity was determined in hemizygous animals of the 6 available lines at neonatal day 12.
[2] Significantly different from wild type, $P < 0.01$.

returned to wild type shortly after weaning. In homozygous F/A-2 mice, arginine concentration also rose significantly after weaning in females ($P < 0.01$), but it remained depressed in males. The relation between intestinal arginase activity and the corresponding arginine concentration in plasma or jejunum is shown in Figure 5B. A sparse-fur phenotype was only seen if intestinal arginase activity exceeded 0.6 U/mg protein or if the circulating arginine concentration dropped below 120 μmol/L. Furthermore, small additional decreases in arginine concentration apparently suffice to cause a pronounced aggravation of the phenotype.

The decrease in jejunal and plasma arginine concentration corresponded directly with increasing arginase concentration in the enterocytes (Figure 5B). Overexpression of arginase in the enterocytes was accompanied by a 30% increase in the arginase product ornithine in jejunum. The accumulation of ornithine was not associated with an increased tissue concentration of the polyamines spermine, spermidine, or putrescine (data not shown). The plasma concentrations of urea, the other arginase product, and of ammonia were not elevated (**Table 2**). As shown in Table 2, the concentration of 2 products of arginine metabolism, creatine and creatinine, known to be sensitive to decreases in plasma arginine (15, 39), was almost halved in both F/A-1 and -2 hemizygous mice. Before weaning, the plasma and tissue concentrations of all measured amino acids except arginine and glycine did not differ in F/A and wild-type mice (data not shown). The increase in plasma glycine concentration, which was similar for both lines and amounted to 90% in the first postnatal week and to 25% and 15% in the second and third weeks, respectively, may have been due to the fact that both arginine and glycine are precursors of creatine.

Effect of arginine supplementation

To show that the F/A phenotype results from arginine deficiency, mice of both F/A-1 and -2 lines were supplemented twice daily with arginine from ND3 on. Fur development and body growth of homozygous F/A-1 animals and hemizygous F/A-2 mice completely normalized and that of homozygous F/A-2 mice partly normalized (Figure 2, C, D, and F). Circulating plasma arginine concentrations in ND10 mice, 6 h after the last injection, were 560, 170, and 90 μmol/L in wild-type, hemizygous, and homozygous F/A-2 animals, respectively ($n = 3$ each). These data show that the transgenic A-I activity in the enterocyte also functions as an effective drain for circulating arginine. Because creatine concentration was halved, we also tested supplementation with creatine, but this treatment was without effect.

Maturation of intestinal mucosa in transgenics

The normal absorption of maternal immunoglobulins (Figure 2H) suggests that the gut of suckling F/A-2 mice functions properly. We assessed periweaning maturation of the enterocytes of the small intestine by monitoring the change in expression of A-II and SI, which normally starts at ND16 and reaches mature levels at ND21, and of ASS, which follows a reciprocal course (12). A-II and SI followed their normal developmental course in homozygous F/A-2 (**Figure 6**A) and F/A-1 mice (data not shown). How-

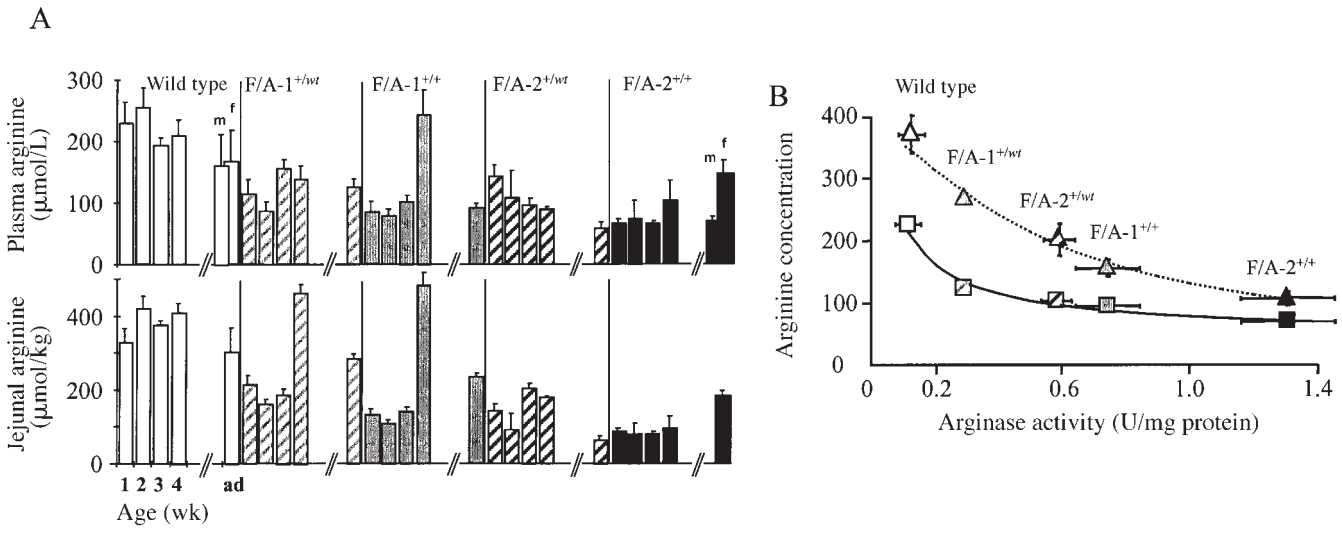


FIGURE 5. A: Arginine concentration in the plasma and jejunum of wild-type (□), F/A-1$^{+/wt}$ (▨), F/A-1$^{+/+}$ (■), F/A-2$^{+/wt}$ (▨) and F/A-2$^{+/+}$ (■) mice at 1–4 wk after birth and as adults (ad). Note that the arginine concentration in transgenic animals is low during the suckling period but recovers after weaning, except in male F/A-2$^{+/+}$ mice. B: Plasma and jejunal arginine concentration as a function of jejunal arginase activity in wild-type, F/A-1$^{+/wt}$, F/A-1$^{+/+}$, F/A-2$^{+/wt}$, and F/A-2$^{+/+}$ mice. On the y axis, plasma (□) and jejunal (△) arginine concentrations are given in μmol/L and μmol/kg wet wt, respectively.

TABLE 2
Products of arginine metabolism in wild-type and F/A transgenic mice[1]

	Wild type	F/A-1	F/A-2
Arginine (μmol/L)	295 ± 27	59 ± 5[2]	63 ± 8[2]
Ammonia (μmol/L)	39 ± 11	40 ± 11	44 ± 14
Urea (mmol/L)	8.1 ± 0.1	8 ± 1	10 ± 2
Creatine (μmol/L)	406 ± 33	218 ± 2[2]	226 ± 70[2]
Creatinine (μmol/L)	18 ± 2	11 ± 1[2]	9 ± 1[2]

[1] $\bar{x}$ ± SEM. Concentrations were measured in serum of 10-d-old animals (n = 3 pools of 3 animals each for each genotype). Note that the arginine concentrations in 10-d-old mice, determined with an amino acid analyzer, differ from the weekly averages in Figure 5, determined with an HPLC analyzer.

[2] Significantly different from wild type, $P < 0.05$ (Dunnett's multiple comparison test).

ever, ASS expression, which had become undetectable in wild types at ND20, was still detectable in F/A-2 mice at this age but not thereafter (Figure 6A). This temporary persistence of ASS expression may reflect a response to the limited bioavailability of arginine, because a similar feedback mechanism was recently reported for rat kidney (40).

We also assessed the pattern of expression of SI and ASS along the villus-crypt axis (Figure 6, B, C, and D). Periweaning maturation of the small intestine started in both wild-type and homozygous F/A-2 mice at ND16 with the appearance of SI at the base of the villi and the disappearance of ASS from this location. The spread of SI expression to the apex of the villi remained tightly associated with the disappearance of ASS but followed a slightly slower course in the F/A-2 mice than in the wild-type animals. ASS was only found to be expressed in immature enterocytes, which, in the ileum, can be identified by the presence of the large supranuclear vacuole (Figure 6C; 41). SI and ASS, therefore, form a useful set of markers to follow enterocyte maturation. Together, the findings indicate that the maturation of the enterocytes in the F/A-2 mice differs from that in wild-type mice only by a slightly protracted time course.

Defective formation of Peyer's patches in homozygous F/A-2 mice

Arginine has been implicated as an important factor in lymphocyte proliferation and function (20). This prompted us to investigate the development of the immune system. At 3 wk, the weight of the spleen and thymus of homozygous F/A-2 mice was less than one-fourth of that of wild-type animals of the same age (**Table 3**). Compared with wild-type pups of the same body weight (ND8), splenic weight was still only 40% of the wild-type weight ($P < 0.01$), whereas thymic weight was not significantly different.

Inspection of the intestines showed that the development of PPs, the first macroscopically visible, morphologic hallmark of the gut-associated lymphoid tissue, was also affected by arginine depletion. In the third postnatal week, 4–7 PPs were always seen protruding from the serosa of the small intestine of wild-type, homozygous F/A-1, and hemizygous F/A-2 mice (**Figure 7**A). However, in a total of 23 small intestines of 18-d-old homozygous F/A-2 animals, only 8 rudimentary PPs were identified macroscopically (Figure 7B) instead of the >92 expected ($P < 0.001$). These PPs were much smaller than those observed in wild-type small intestines (Figure 7, E and F). In homozygous F/A-2 pups that were supplemented with arginine, a normal number of normally sized PPs were found (Figure 7C). No such effect was seen after treatment with saline or creatine. Normal development of PPs is, therefore, critically dependent on arginine availability. To establish whether PP organogenesis was disturbed, we performed whole-mount immunostaining of the small intestine of 6-d-old pups for the presence of VCAM-1, the first marker of PP development (42). This endothelial receptor for the integrin α4 is essential for lymphocyte homing to the PP. The number and size of VCAM-1–positive cell clusters (4–7) in the small intestines of homozygous F/A-2 mice were indistinguishable from those in wild-type animals, showing the presence of intact PP anlagen (Figure 7, D and G). In homozygous F/A-2 mice, PPs became macroscopically identifiable at 6–7 wk after birth. These data show that development of PPs in F/A-2 mice is temporarily suspended but not abolished. The development of the mesenteric and peripheral lymph nodes is undisturbed in homozygous F/A-2 mice.

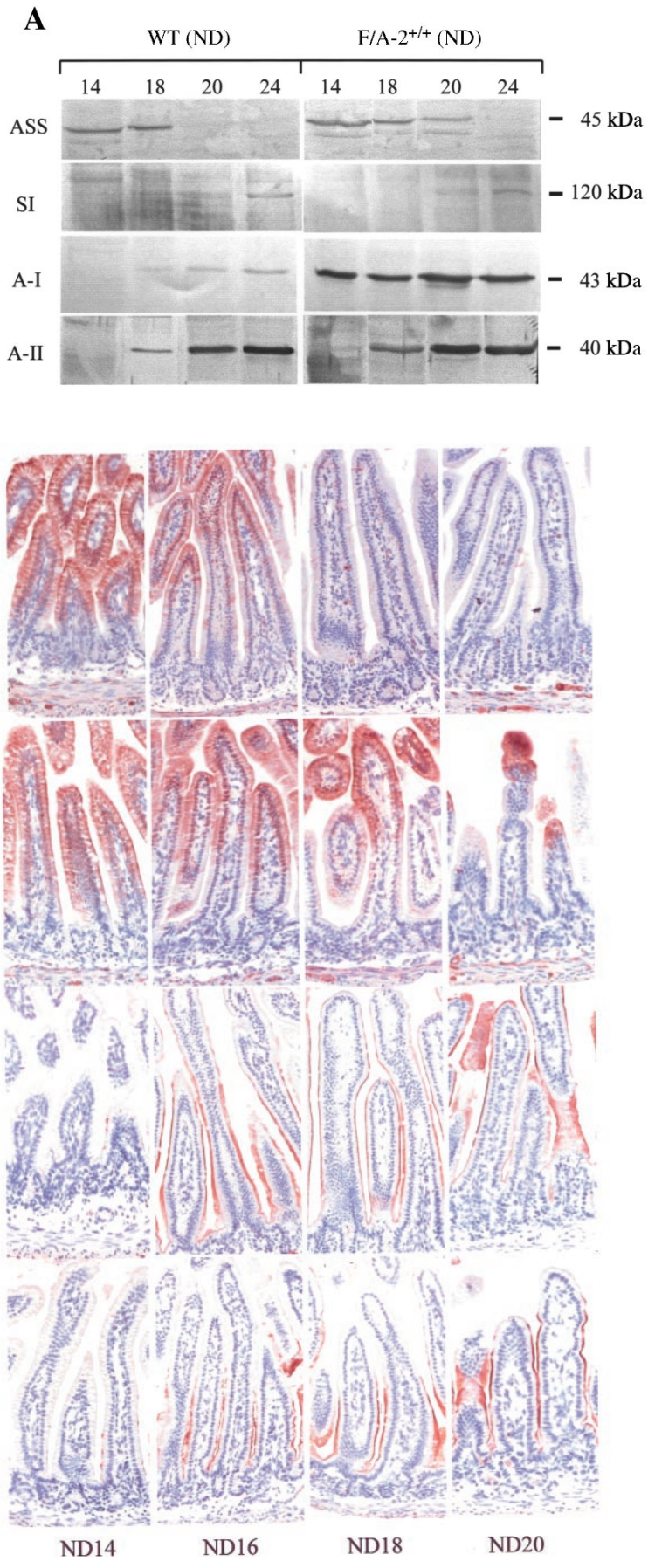
To reveal a possible mechanism for the effect of arginine on PP development, we investigated mice carrying null mutations of the 3 isoforms of the NOS gene (43–45) or the *spf-ash* mutation in the OTC gene. At ND18, the expected number and size of PPs were found in *spf-ash*–, nNOS-, iNOS-, and eNOS-deficient mice. This observation shows that the hampered development of PPs is not due to deficient citrulline or nitric oxide production by any of these enzymes. In addition, the normal number and size of PPs in *spf-ash* mutants suggest that the failing development of PPs in F/A-2 homozygotes is not caused by runting. Together, the data suggest that the defective development of PPs in F/A-2 mice is caused by arginine deficiency rather than by deficiency or aberrant concentrations of one of its metabolic products.

DISCUSSION

Requirements for the development of the F/A phenotype

Phenotypic characteristics of transgenic mice that express arginase in their enterocytes include a temporary retardation of body and fur growth and of the development of the immune system, including PPs. As shown by the normal arginine concentration and phenotypic appearance of lines F/A-3, -4, -5, and -6, expression of the transgene in all enterocytes is necessary to elicit the characteristic phenotype. As shown by the differences between hemi- and homozygous F/A-1 and -2 pups, the severity of the phenotype depends on the level of expression of transgenic A-I in the enterocytes, that is, the degree to which cytosolic arginase interferes with arginine metabolism. This assessment is underscored by the transient nature of the F/A-1 and -2 phenotype, which ameliorates after the arginine synthesis in the small intestine stops (12). However, the results of the arginine injections show that the A-I–overexpressing enterocytes also function as an effective arginine drain. Because F/A-2 adult males have a lower circulating concentration of arginine than do females, they are apparently more sensitive to this effect than females are. We do not know whether the F/A phenotype will also develop if transgenic arginase is expressed in another tissue than the gut. If not, it would imply that the suckling gut requires a local source of arginine that is insufficiently met by the supply via the milk. We are exploring this issue.

The graded phenotype of the F/A transgenics reveals a relation between circulating arginine concentration and retardation of growth and development. The transient sparse-fur phenotype of F/A mice is also seen in OTC-deficient *spf-ash* mice. At 3 wk, plasma and intestinal arginine concentration in homozygous



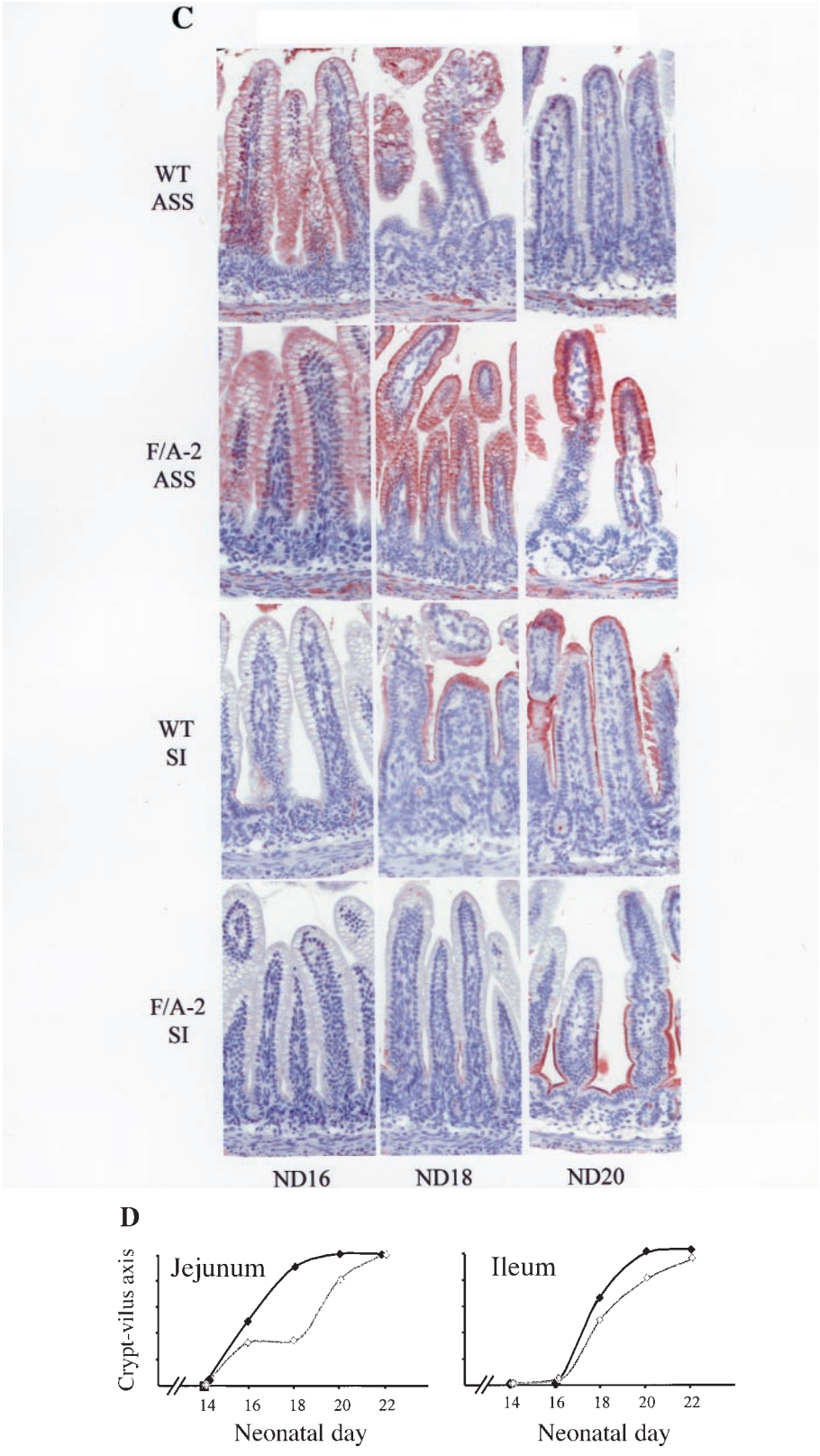


FIGURE 6. A: Western blots of jejunal tissue of wild-type (WT) and F/A-2$^{+/+}$ mice at neonatal days 14 (ND14), 18, 20, and 24, probed with polyclonal antibodies against argininosuccinate synthetase (ASS), sucrase-isomaltase (SI), arginase I (A-I), and A-II. kDa, kilodaltons. B and C: Expression of ASS and SI along the crypt-villus axis of the jejunum (B) and ileum (C) of WT and F/A-2$^{+/+}$ mice (+/+) at ND14 (jejunum only), 16, 18, and 20. All sections were counterstained with hematoxylin. D: Migration of the boundary between immature (ASS-positive, SI-negative) and mature (ASS-negative, SI-positive) enterocytes along the crypt-villus axis in the preweaning period in WT (◆), and F/A-2 (◇) mice. Note the identical onset, but slightly protracted progress, of enterocyte maturation in homozygous F/A-2 mice.

TABLE 3
Splenic and thymic weights in wild-type and homozygous F/A-2 mice[1]

	Wild type (ND21)	F/A-2 (ND21)	Wild type (ND8)
Body weight (g)	13.5 ± 1.0	4.7 ± 0.3	4.8 ± 0.2
Splenic weight (mg)	67 ± 10 (0.5)	15 ± 9[2,3] (0.3)	38 ± 1 (0.8)
Thymic weight (mg)	79 ± 7 (0.6)	17 ± 4[2] (0.4)	24 ± 1 (0.5)

[1] $\bar{x}$ ± SEM; *n* = per group. Organ weight as a percentage of body weight in parentheses. ND, neonatal day.
[2] Significantly different from wild-type mice at ND21, $P < 0.01$.
[3] Significantly different from weight-matched (ND8) wild-type mice, $P < 0.01$.

spf-ash mice are ≈120 μmol/L (46, 47). Combining these data with our own, we conclude that the sparse-fur trait develops when plasma arginine in the suckling period drops below 120 μmol/L. Hampered muscle and immune development is seen only in homozygous F/A-2 mice and, therefore, develops when plasma arginine concentration drops below 80 μmol/L. The lack of a linear relation between intestinal arginase concentrations and plasma arginine concentrations in the respective transgenic lines may be due to induction of arginine biosynthesis elsewhere, eg, in the kidney (40). Given the high Michaelis constant of A-I, which is in the millimolar range, such an extraintestinal biosynthetic capacity may be sufficient to blunt the effect of increasing levels of arginase in the enterocytes.

The lack of normal body growth in homozygous F/A-2 mice appears to reflect impaired muscle development rather than starvation. In fact, the normal concentration of serum IgG, which is actively taken up from the milk (48) by specific neonatal Fc receptors on the intestinal epithelium (38), shows that milk intake and absorption in F/A-2 mice is normal. The findings that the plasma and tissue concentrations of all measured amino acids except arginine, ornithine, and glycine are normal and that the impaired growth of fur and muscle and the impaired PP development are alleviated by arginine injections further support the hypothesis that the observed developmental disorders result solely from an arginine deficiency.

Causes of the development of the F/A phenotype

Arginine is a precursor for the synthesis of proteins, creatine, agmatine, nitric oxide, and polyamines and an intermediate in the detoxification of ammonia. We therefore evaluated these pathways for a possible cause of the F/A phenotype. Because the concentration of ammonia and polyamines in F/A-2 mice is normal, these factors can be excluded. The observed tissue arginine concentration in the F/A-2 transgenics exceeds the Michaelis constant of arginyl–transfer RNA (tRNA) synthetase (EC 6.1.1.19) for arginine (≈20 μmol/L; 49); thus, incomplete charging of arginyl-tRNA as the limiting factor for protein synthesis appears less likely. However, a more important argument than cellular arginine concentration is that incomplete charging of arginyl-tRNA should preferentially affect the synthesis of proteins with a very high arginine content, especially if the arginines are adjacent (50). An example of such an extremely arginine-rich protein is trichohyalin, a major intermediate filament–associated protein in the inner root sheath and medulla of hair follicles with 21.5% of its amino acids consisting of arginines, including 70 diplets and 14 triplets (36). Unexpectedly, no decrease in trichohyalin content was found in tissue sections and Western blots of F/A-2 skin, indicating a similar balance in transgenic and control skin between synthesis and degradation of this extremely arginine-rich protein. Because it appears unlikely that protein degradation is decreased in runting animals,

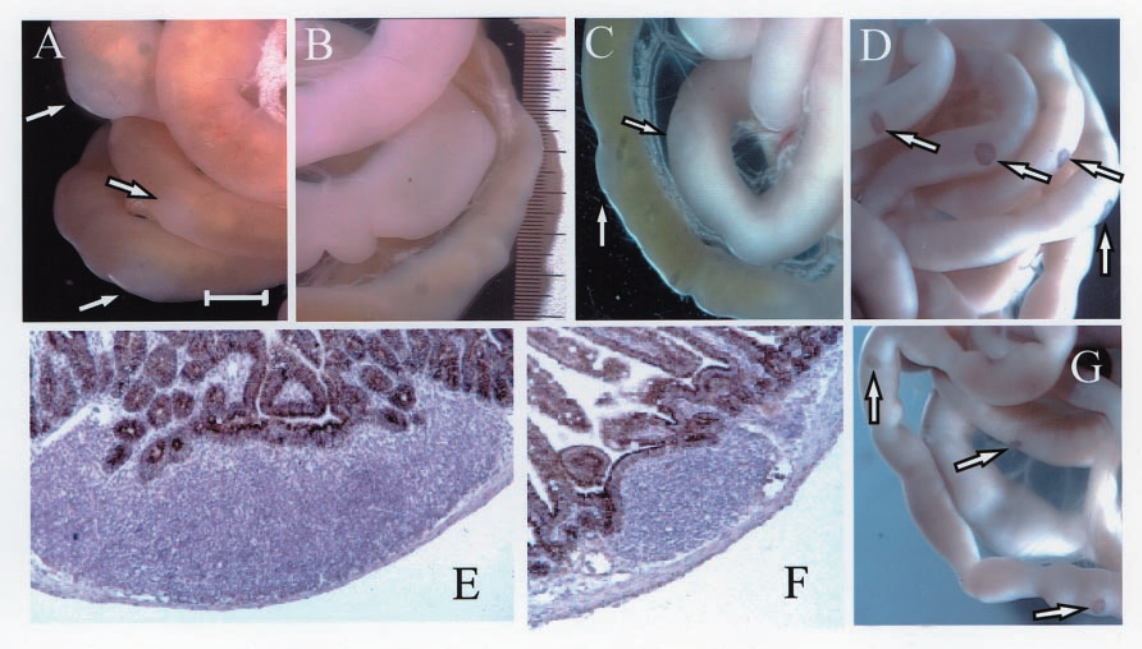


FIGURE 7. A and B: Peyer's patch (PP) development in the small intestine of a wild-type (A) and an F/A-$2^{+/+}$ (B) transgenic mouse at neonatal day 18 (ND18), with arrows pointing at PPs. PPs were not macroscopically identifiable in F/A-$2^{+/+}$ mice. C: Arginine injections normalize the appearance of PPs in F/A-$2^{+/+}$ mice (arrows). D and G: Whole-mount immunohistochemical staining of the small intestine of an ND6 wild-type (D) and an F/A-$2^{+/+}$ (G) mouse for the presence of vascular cell adhesion molecule 1–positive PP anlagen (arrows). Note the presence of anlagen in both genotypes. E and F, hematoxylin- and azophlochsin-stained sections of a typical PP in a wild-type (E) and an F/A-$2^{+/+}$ (F) mouse. The bar in A represents 0.6 mm in A, B, and C; 3 mm in D and G; and 75 μm in E and F.

we interpret this finding as evidence that arginine deficiency does not exert its effects on fur development and, by inference, neonatal growth at the level of arginyl-tRNA synthesis.

Histologically, the muscle fibers of F/A-2 mice are 2–3-fold smaller than those of control animals and suggest delayed development. Although a positive effect of creatine supplementation on muscle mass is disputed, we tested it because it was shown that metabolic deficiency of creatine can be corrected by substitution (51). From the absence of an effect, we conclude that the F/A phenotype does not result from a creatine deficiency. We therefore do not know how arginine regulates normal muscle growth. However, it is noteworthy that the muscular phenotype that develops as a result of α7-integrin deficiency also becomes manifest soon after birth (52).

The decreased spleen weight and the tardy development of PPs indicate that the selective decrease in circulating and tissue arginine concentrations retards the development of the immune system. In accordance with the relatively normal ratio of the thymus to body weight, our preliminary data indicate that arginine deficiency primarily affects B lymphocyte development. To our knowledge, a direct stimulatory effect of a common nutrient such as arginine on immune development in vivo has not yet been shown, although the therapeutic potential of arginine as an immunonutrient in enteral and parenteral nutrition in a clinical setting was already claimed 21 y ago (53). Because nontoxic concentrations of nitric oxide are thought to enhance T helper 2–dependent immune responses (54), PP development was investigated in mice deficient in nNOS, iNOS, or eNOS. In these mice, normal PPs were found, suggesting that the effect of arginine deficiency is not exerted via any of these enzymes separately. Unfortunately, mice deficient in all 3 NOS isoforms are not available to conclusively prove or disprove nitric oxide involvement.

Together, our findings indicate that the F/A phenotype develops as a result of a deficiency of arginine itself, rather than from a deficiency of downstream products of arginine metabolism. Clearly, identification of the pathway that is primarily affected by arginine deficiency in the suckling period will be the next target of research. In this respect, the biology of PP development appears particularly promising, because homozygous F/A-2 mice share the combination of normal organogenesis, disturbed maturation of PPs, and normal lymph node development with mice deficient in tumor necrosis factor α and tumor necrosis factor α receptor 1 (55, 56). Our preliminary observation that F/A-2 mice have a drastically diminished number of mature B cells in spleen and intestine may represent another lead, because the development of PPs, follicle-associated epithelium, and M cells was also found to be impaired in mice that lacked B cells (57).

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