Implications of arginine deficiency for growth and organ maturation. Studies on hair, muscle, brain and lymphoid organ maturation

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Implications of arginine deficiency for growth and organ development

Studies on hair, muscle, brain and lymphoid organ maturation

Wouter J. de Jonge
Implications of arginine deficiency for growth and organ maturation

Studies on hair, muscle, brain and lymphoid organ maturation

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ten overstaan van een door het college voor promoties
ingestelde commissie, in het openbaar te verdedigen
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Arginine biosynthesis and metabolism

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Abbreviations and enzymes with enzyme codes used:

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<th>Enzyme</th>
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<td>SI</td>
<td>[EC 3.2.1.48-10]</td>
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</tr>
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EC = enzyme code, n.a. = not assigned
Introduction

*1-Biosynthesis of arginine*

1.1-Intestinal citrulline biosynthesis

The organ perfusion studies by Windmueller and Spaeth \(^1,2\) established that de novo synthesis of citrulline, as well as ornithine and proline, takes place in the small intestine (Fig. 1). Citrulline synthesis requires \(\text{NH}_3\), \(\text{CO}_2\), and ornithine. The \(\text{NH}_3\) is derived from glutamine via the action of glutaminase. Together with bicarbonate, \(\text{NH}_3\) can form carbamoylphosphate by the action of carbamoylphosphate synthetase (CPS-1; EC 6.3.4.16), an enzyme that requires N-acetylglutamate for activity. The carbamoyl group is transferred to ornithine to form citrulline in a reaction catalyzed by ornithine transcarbamoylase (OTC; EC 2.1.3.3). In addition to glutamate, proline can function as a precursor for the intestinal ornithine synthesis (Fig. 1) \(^3\), via the formation of P5C by proline oxidase, and the subsequent non-enzymatic conversion into L-glutamyl-\(\gamma\)-semialdehyde \(^4\).

The enzymes involved in citrulline synthesis, phosphate-dependent glutaminase, OAT, and aspartate aminotransferase are found in a variety of tissues \(^5,6\), but CPS I, OTC, and N-acetyl glutamate synthetase expression are restricted to the liver and intestinal mucosa \(^7\). P5C synthetase, which catalyzes the multistep conversion of glutamate via glutamyl-\(\gamma\)-semialdehyde into P5C \(^4\), is present almost exclusively in the small intestinal mucosa, with only trace amounts in other tissues \(^4\). P5C is, in turn, converted into ornithine by OAT. For this reason, the intestine is the only organ that has the capacity of net production of ornithine. In (most) other organs, including liver, OAT converts ornithine into glutamate. Human patients suffering from a deficiency in P5C synthetase develop hyperammonemia and hypoornithinemia \(^8\). In addition, these patients have decreased proline levels, implying that P5C-S may also have a P5C-R like activity. The essential role of the intestine to provide citrulline for arginine biosynthesis elsewhere, is also demonstrated by the arginine deficiency that results from inhibition of intestinal
citrulline synthesis, as a result of inhibition or deficiency of OTC $^9,10$, OAT $^{11,12}$, or by massive resection of the small bowel $^{13}$.

In intestinal tissue, arterial and luminal glutamine is the main metabolic contributor of intestinal citrulline biosynthesis $^2$. The magnitude of the role of proline in citrulline synthesis is disputed. In pigs, there is no arterial uptake of proline by the gut $^{14}$, suggesting that only dietary proline is metabolized into citrulline. Indeed, a prominent role for proline is suggested by the observation that, at least in pigs, proline is among the most abundant amino acids in the sow’s milk $^{14}$.

Fig. 1: Scheme of the relation between glutamine and the ornithine cycle. Enzymes that catalyze the reactions are: 1- phosphate-dependent glutaminase [EC 3.5.1.2], 2- pyrroline-5-carboxylate synthetase [EC n.a.], 3- ornithine aminotransferase [EC 2.6.1.13], 4- carbamoyl-phosphate synthetase [EC 6.3.4.16], 5- ornithine transcarbamoylase [EC 2.1.3.3], 6- argininosuccinate synthetase [EC 6.3.4.5], 7- argininosuccinate lyase [EC 4.3.2.1], 8- hepatic arginase [EC 3.5.3.1] - kidney-type arginase [EC 3.5.3.1], 9- proline oxidase [EC n.a.], 10- aspartate aminotransferase [EC 2.6.1.1]. Enzymes 6, 7 and 8 are cytosolic, 1-5 and 9 are mitochondrial, and enzyme 10 is both cytosolic and mitochondrial.

Though proline oxidase (PO) is reported to be present mainly in liver and kidney $^{6,15}$, other groups have detected a relatively high level of PO activity in the intestinal
mucosa of pig and rat \textsuperscript{16,17}. This difference has been attributed to instability of the enzyme during homogenization of, specifically, mucosal tissue \textsuperscript{6}.

\section*{1.2- Arginine biosynthesis}

\subsection*{1.2.1-Renal arginine biosynthesis.}

In the adult kidney, arginine is made endogenously by converting citrulline into arginine, mainly in the renal proximal tubules \textsuperscript{1,18}, via the cytosolic enzymes ASS and ASL. The kidney accounts for 60\% of arginine synthesis from circulating citrulline \textsuperscript{19} (see section 2.2.2). Although the kidney expresses CPS-1, (unpublished observation), as well as glutaminase and glutamate dehydrogenase (GDH) \textsuperscript{20,21} in the preweanig period, the adult kidney lacks the capacity for carbamoylphosphate synthesis.

The citrulline that is metabolized into arginine by the kidneys derives primarily from the intestine \textsuperscript{1,22}. The amount of citrulline taken up for arginine synthesis has been shown to be proportional to the amount released by the intestine \textsuperscript{1,23}. Furthermore, a positive correlation between renal citrulline uptake and arginine production has been shown in humans \textsuperscript{24}, sheep \textsuperscript{25} and rats \textsuperscript{26}. The capacity of the kidney to synthesize arginine exceeds the capacity of the intestine to synthesize citrulline several-fold, indicating that renal arginine synthesis depends on intestinal citrulline synthesis \textsuperscript{27}. In addition, the renal arginine flux is highly regulated: renal ASS and ASL mRNA as well as activity are upregulated during a high protein diet \textsuperscript{28}, upon starvation \textsuperscript{29}, or upon an acute decrease in circulating arginine levels under pathological conditions \textsuperscript{30}.

Next to the cytosolic arginine-synthesizing enzymes, the kidney expresses A-II (EC 3.5.3.1) \textsuperscript{31}, but the expression of arginase, ASS and ASL is segregated in different parts of the nephron. Arginine synthesis is restricted to the upstream portions of proximal convoluted tubules, whereas arginase activity is present mainly in the cortical and outer medullary portions of straight proximal tubules. Hence, there is little or no co-expression of these enzymes in the same cell \textsuperscript{32}, allowing for net renal arginine biosynthesis from the citrulline reabsorbed from glomerular
filtrate in proximal convoluted tubuli cells, while in straight tubuli, arginase-derived urea passively diffuses into the luminal fluid entering Henle's loops \(^3^2\).

### 1.2.2-Extra-renal arginine biosynthesis

\(\text{ASS mRNA}\) is expressed in the neurons of the myenteric plexus of the adult small intestine \(^3^3\), suggesting the existence of a neuronal arginine-citrulline cycle for regeneration of arginine for nitric oxide synthesis. Such a cycle has been described in several other nitric oxide producing cell-types, like endothelial cells \(^3^4,3^5\), macrophages \(^3^6\), and neurons \(^3^7,3^8,3^9\). The picture therefore emerged that citrulline synthesis is confined to the small intestinal enterocytes, whereas arginine biosynthesis occurs in many tissues, the kidney being the most prominent. Presumably, the co-localization of \(\text{ASS}\) and \(\text{ASL}\) with \(\text{nNO S}\) serves to assure that cellular NO synthesis becomes independent of circulating arginine. Thus, \(\text{ASS}\), \(\text{ASL}\), and \(\text{nNOS}\) immune reactivity (or NADPH-diaphorase activity) have been shown to colocalize in the central nervous system of rat \(^3^7,3^8\) and pig \(^3^9\). Whether \(\text{ASS}\) and \(\text{ASL}\) indeed colocalize with \(\text{nNOS}\) in the intestinal neurons and whether this renders these neurons independent of extracellular arginine through intraneuronal recycling of \(L\)-citrulline to \(L\)-arginine, is not clear. In vitro, inhibitory neuromuscular transmission in murine proximal colon was stimulated by arginine, but not by citrulline alone (0.1-2 mM), which in principle indicates that enteric nerves were not able to convert citrulline into arginine \(^4^0\). In contrast, when nitrergic transmission was blocked by the NOS antagonist L-NAME, sustained neuronal exposure to citrulline (0.2 mM) reversed the effects of L-NAME \(^4^0,4^1\), revealing the capacity of enteric nerves to recycle L-citrulline to L-arginine to sustain NO synthesis. Most likely, enteric neurons thus do not depend on extracellular arginine for generation of NO. Measurement of the inhibitory component of the intestinal enteric neuron system, evoked by NANC neurons, in organ bath experiments using intestinal tissue from our arginine-depleted transgenic F/A mice \(^4^2\), may provide evidence for the (in)dependence of enteric neurons on extracellular arginine availability.
Similar to neurons, macrophages, activated by LPS or IFNγ, have been shown to express ASS, ASL, and A-I and A-II. Despite the upregulation of ornithine cycle enzymes required for regeneration of arginine from citrulline, macrophages remain dependent on extracellular arginine, as is shown by the upregulation of their arginine transport capacity (via the enhanced expression of CAT-2) in conjunction with iNOS induction. The upregulation of either A-I (A-II is not upregulated) or iNOS may be subject to the nature of the inflammatory response: the upregulation of A-I may be directed by Th2-derived anti-inflammatory cytokines, whereas Th1-associated pro-inflammatory cytokines induce iNOS expression. In section 4, the potential role of macrophage A-I induction in counteracting the macrophage NO output is further discussed.

1.3-Hepatic arginine metabolism

The liver does not participate in whole-body arginine synthesis, despite very high levels of ASS and ASL. This is because the very high arginase content of hepatocytes prevents the net production of arginine, which emphasizes the importance of the liver for urea production (ammonia detoxification) rather than for arginine biosynthesis. Moreover, the liver lacks expression of the high affinity cationic amino acid transporter (CAT-1), which is required for efficient hepatic import or export of arginine from the circulation. In concert with the argument that the liver does not contribute arginine to the circulation, patients with inherited ornithine cycle deficiencies, who need supplementation of arginine, continue to do so after transplantation of the liver.

1.4-ontogeny of arginine metabolism

In rapidly growing suckling rats, endogenous arginine biosynthesis is crucial to compensate for the insufficient supply of arginine via the milk. During the suckling period, the kidney has a limited capability for the synthesis of arginine. Evidence is accumulating that, in mammals, the intestine rather than the kidney plays a major role in arginine biosynthesis in this period. Thus, the enterocytes of
the small intestine express CPS, AGA synthetase, OTC, ASS and ASL \(^\text{11,27,55,56}\), i.e. the enzymes required for arginine production from glutamine\(^3\), but do not express arginase\(^33\). Similarly, enzymes that convert glutamine and proline to ornithine, glutaminase, P5CS and OAT, are all present in concentrations well above adult levels in the period before weaning\(^\text{57-60}\), whereas the activity of pyrroline-5-carboxylate reductase (P5CR), the enzyme that can divert pyrroline-5-carboxylate towards proline, shows a reciprocal pattern\(^56\). The intestine appears to play a similar role in human neonates as well, as destruction of the enterocytes in necrotizing enterocolitis also results in a selective decrease in circulating arginine\(^61\). In mammals, ASS and ASL, disappear from the enterocytes in the periweaning period, concurrent with the appearance of endogenous arginase\(^33\). After weaning, the role of the gut therefore becomes confined to citrulline biosynthesis.

The ontogeny of arginine metabolism in intestinal epithelium is associated with its maturation, as expression of ornithine cycle enzymes changes during migration along the crypt-villus axis. From the formation of villi onwards, glutaminase\(^62\), OAT, CPS and OTC mRNA are concentrated near the base of the villus, whereas ASS and ASL mRNA are only present near the top\(^33,42\). We have suggested that this baso-apical difference in gene-expression relates to the time after stem-cell division, and migration of the enterocytes along the crypt-villus axis\(^33\). The enterocytes near the top of the villus are "older" than those at the base, implying that the presence of OAT, CPS and OTC protein in all villus enterocytes, including those at the top of the villus, is due to the half-life of these proteins that were synthesised when the enterocytes were still localized at the base of the villus. Because their mRNAs are only present in the apical enterocytes, ASS and ASL apparently become expressed only several days after enterocyte cell division and/or migration along the villus axis. The transit time of the enterocytes between the crypt and the top of the villus, which decreases from 11-14 days in the first postnatal week, to 2-3 days after weaning\(^63,64\), may thus explain the difference between the pre- and post weaning expression pattern\(^33\). In contrast with this hypothesis, however, a decrease in enterocyte migration rate, through the transgenic
overexpression of E-cadherin, is not accompanied by a change in distribution of the terminal differentiation marker liver-type FABP along the crypt-villus axis, suggesting that differentiation, at least for liver-type FABP, is largely cell nonautonomous.

Together, the observed expression pattern of ornithine cycle enzymes suggests the existence of a metabolic compartmentation of citrulline synthesis in the enterocytes at the base of the villus and arginine synthesis in the top of villus enterocytes. Such a two-compartment system along the villus axis may enable the gut to simultaneously meet the needs for the synthesis of citrulline and arginine during the suckling period. Further examination of the expression of arginine transporters, or measurement of arginine and citrulline concentration in the two compartments should confirm this hypothesis.
2-Dietary demand of arginine

2.1-Dispensable and indispensable amino acids

Protein requirements have long been assessed by the sum of requirements of the essential amino acids and the nitrogen from non-essential amino acids \(^66\). By preparing diets containing mixtures of amino acids as the only sources containing dietary nitrogen, the quantitative requirement for each amino acid was determined. In rats, it was found that of the 20 amino acids, that are incorporated into protein, 10 could not be excluded from the diet: valine, leucine, isoleucine, methionine, threonine, lysine, phenylalanine, tryptophan, histidine, and arginine \(^67\). Excluding any one of these essential amino acids, except arginine, resulted in weight loss, and eventually death. However, although exclusion of arginine led to decreased weight gain, but allowed the animals to survive \(^67\). The endogenous synthesis of arginine was apparently adequate for survival, but not for optimal growth. The classification of arginine as being a dispensable or indispensable amino acid is therefore a matter of definition, as arginine can be synthesized endogenously, but in limited quantities only. Irrespective of definitions, the limited endogenous biosynthetic capacity is most obvious under conditions of increased demand for- or catabolism of arginine \(^67\). Indeed, arginine supplementation has been shown to ameliorate recovery and reduce hospital state under catabolic conditions, such as sepsis or trauma \(^68,69\), also reviewed in \(^70-72\).

2.2-The homeostasis of creatine reflects a delicate arginine balance

The highest level of creatine is found in muscle, Sertoli cells \(^73\), and brain \(^74\), where it plays an essential role in the energy metabolism \(^74,75\). The biosynthesis of creatine first comprises the formation of guanidino acetic acid (GAA) from L-arginine and glycine by L-arginine-glycine amidinotransferase (AGAT EC 2.1.4.1., Fig. 2), followed by the transfer of a methyl group by S-adenosylmethionine:guanidinoacetate methyltransferase (GMT) \(^75\). The delicate balance of the dietary supply and endogenous biosynthesis of arginine with its daily
expenditure is nicely illustrated by the direct relation between the dietary intake of arginine and the body pool size of its product creatine. A young adult male excretes on average of 23 mg (0.2 mmol) of creatinine per kg body weight per day. Thus, 0.2 mmol arginine per kg body weight is required for creatine synthesis. The recommended daily allowance for protein is 0.8 gram protein per kg of body weight, supplying 0.25 mmol of arginine per kg of body weight. Creatine synthesis alone, therefore, requires 80% of the daily intake of arginine. This tight balance may well explain why the creatine pool size is influenced significantly by changes in the quantities of arginine, glycine or creatine in the diet.

Figure 1: Metabolic routes of creatine synthesis from arginine. Enzymes that catalyze the indicated reactions are given in italics. AGAT transamidinates the guanidino group of arginine to glycine, but also to the other substrates indicated. Proposed transamidination substrates: glycine (Gly), $\delta$-aminopropionic acid (GPA), $\gamma$-aminobutyric acid (GABA), $\beta$-aminovaleric acid ($\delta$-AVA), and products: $\beta$-guanidinopropionic acid (GPA), $\delta$-guanidinobutyric acid (GBA), and $\delta$-guanidinovaleric acid (GVA). Products formed by reactive oxygen (see text): guanidinosuccinic acid (GSA), methylguanidine (MG) and guanidine (G). GSA and MG are most probably formed from argininosuccinate (AS) and CTN, respectively, via a reaction with a reactive oxygen species (see also section 6).

2.3-Arginine demands in growing mammals
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Davis et al compared the supply of amino acids via the milk and their accumulation in body proteins of rat to estimate the degree to which each amino acid in the milk is utilized for protein accretion. The essential amino acids were all utilized for protein synthesis with efficiencies of around 75%, indicating that only 25% of amino acids are degraded in the suckling period \(^5^4\). This finding corroborates an earlier finding that excretion of urea in this period is very small \(^7^9\), see also chapter IV. Similar findings were reported for other species like pig, calf, sheep, chicken and human \(^8^0,8^1\). However, 170 and 270% of ingested arginine and glycine, respectively, i.e. the precursors of creatine, was found in the lean body mass, indicating that they were actively synthesized.

2.4-Species differences in arginine requirement and biosynthesis
As described above, it has been shown that dietary arginine is required for optimal growth of species like rodents and pigs, but for obligate carnivorous species like cats and mink, it is an essential amino acid \(^2^8\), due to the virtual absence of P5C synthetase and OAT in the intestine of these carnivores \(^8^2-8^5\) (see also 3.1). The resulting deficiency in ornithine biosynthetic capacity renders their ornithine availability directly dependent on dietary arginine. The result of this can be devastating: cats develop hyperammonemia and show clinical symptoms of ammonia toxicity within 2 hours after consumption of an amino acids containing meal without arginine \(^8^6,8^7\), whereas non-carnivorous species show only minimal signs of intoxication when fed arginine-free diets \(^8^8\). Since ornithine and citrulline occur only in very low concentrations in ordinary dietary proteins, feline species depend on dietary arginine \(^6^7,8^7\) to spike the ornithine cycle and maintain high capacity for ammonia detoxification. Thus, in carnivorous species, dietary arginine supply can be regarded critical for maintaining the urea cycle capacity for detoxifying ammonia \(^8^9\).
Introduction

3-inborn errors of metabolism leading to congenital arginine deficiency

3.1 Ornithine aminotransferase

The small intestinal mucosa is the only site of synthesis of pyrroline-5-carboxylate (P5C) from glutamate or proline\(^4,28\) (Fig. 1). P5C is a precursor for the non-enzymatic conversion to glutamate-\(\gamma\)-semialdehyde, which, in turn, is the substrate of OAT for ornithine formation. Carnivorous species, like the cat, have only 18% (per gram of mucosa), and 5% (per kilogram body weight) of intestinal P5C activity compared with rat. This limitation in the de novo synthesis of ornithine forms the metabolic basis for the severe hyperammonemia found in cats fed an arginine-deficient diet\(^90\).

Patients that lack OAT\(^91,92\) develop hyperornithemia and gyrate atrophy (GA), which is characterized by night blindness and diminished peripheral vision in the first and second decades of life, followed by progression to total blindness. In GA patients, hyperornithemia is associated with a reduction in the formation of creatine\(^93\), via inhibition of AGAT activity by the excess ornithine\(^94\). This creatine deficiency has been proposed to account for pathophysiology of the disease\(^95\), as the mass of skeletal muscle tissue in GA patients is only 24% of that of controls. Unexpectedly, arginine was found to be an essential amino acid in young growing patients with this inborn error\(^93\). Mice, in which the OAT gene was genetically targeted, can serve as an animal model for GA\(^12\). In young mice with this deficiency, hypo-ornithemia (56 \(\mu\)M, 50% of normal) and –argininemia (26 \(\mu\)M, less than 10% of normal), and an impaired growth rate are seen, demonstrating the requirement for ornithine biosynthesis from pyrroline-5-carboxylate at this age\(^12\). Interestingly, OAT-deficient mice develop hyperornithemia (>1mM) as adults, elegantly demonstrating that the OAT enzyme is directed towards ornithine synthesis in the neonatal small intestine and liver, but is reversed towards P5C.
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synthesis in many adult tissues. A similar phenomenon seems to occur in human patients as they grow older. 91.

3.2 Ornithine transcarbamoylase

The human deficiency of OTC, the gene being located on the X-chromosome, was first described in 1962. 96 It is regarded as the most common ornithine cycle deficiency in man. 10 Affected male infants generally die in the first few weeks after birth, due to acute hyperammonemic coma, though some chronic cases are known. 97 Well studied murine models of OTC deficiency are the sparse-fur (spf) and sparse-fur with abnormal skin and hair (spf-ash) mice. These mutant mice suffer from congenital hyperammonemia and hypoargininemia and can serve as a useful model to study the neurochemical consequences of congenital urea cycle disorders, including hyperammonemic encephalopathy. Spf-ash mice are characterized by retarded body- and fur growth, and an excitable hyper-reactive temperament. 10,98 Biochemically, these mice are characterized by a significantly elevated orotic acid excretion, elevated serum ammonia, glutamine and tryptophan levels, 10,99 and a decrease in all other amino acid levels. 99 Especially the ornithine cycle intermediates arginine, ornithine and citrulline are decreased to 20-50% of normal. 99

In children, encephalopathy as a result of chronic hyperammonemia often seen in OTC deficiency is associated with changes in cerebral neurotransmitter systems. 100 Similarly, Spf mice display behavioral abnormalities and brain damage, 101 which can be attributed to alterations in a number of neurotransmitter systems, (NO, ATP, and glutamate), ammonia, or the tryptophan-derived excitotoxin quinolinic acid. 104 Moreover, Spf mice have an elevation in the levels of neurotoxic guanidino compounds, such as GSA. 105 Recently, we described behavioral aberrations, similar to those in Spf mutants, in our mouse model of arginine deficiency (F/A mice). 42 We showed that this model is more selective in its amino acid deficiency than Spf mutants, as F/A mice have normal levels of ammonia, urea, and all amino acids except arginine. Although, the formation of GSA
is increased in both F/A and Spf mice, this compound is not increased in the brain of F/A mice, whereas it is in Spf-ash mice. Hence, a comparison of the two models suggests that the deficiency of arginine, or its direct metabolites (NO, agmatine or creatine), is the determining factor in the development of neuromotor deficits in both models. This conclusion would also justify the arginine treatment of OTC-deficient hyperammonemic patients.

### 3.3 Argininosuccinate synthetase

Citrullinemia is an autosomal recessive disorder, caused by a deficiency of argininosuccinate synthase (ASS). It is characterized by elevated levels of blood citrulline, ammonia, and orotic aciduria. Patients suffer from a disturbance of consciousness and coma, and most die with cerebral edema within a few years of onset. In man, ASS deficiency can manifest as a neonatal (CTLN1), or an adult-onset form (CTLN2) and as an asymptomatic form. CTLN1 is associated with mutations in the ASS gene, leading to due to ASS enzyme defects, whereas CTLN2 is a form of the disease that is defined by a decrease in ASS protein, but with normal kinetic properties. The protein being effected in CTLN2 may be a calcium-dependent mitochondrial transporter with a role in urea cycle function. The phenotype of ASS-deficient mutant mice closely resembles that of humans who lack the ASS enzyme. Homozygous mutant animals develop high levels of blood citrulline, become hyperammonemic, and die within one or two days after birth.
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4-Metabolism of arginine

4.1-Intestinal utilization of glutamine and arginine

The small intestine of the adult rat extracts 25-33% of arterial glutamine in a single pass, which accounts for 30% of whole body glutamine utilization \(^{17,111}\). Until the pioneering work of Windmueller on glutamine utilization by rat jejunum \(^2\), it was tacitly assumed that all dietary amino acids absorbed by the small intestinal mucosa entered the portal circulation intact and became available for extra-intestinal tissues \(^{17}\). However, intraluminally delivered glutamine is utilized extensively by the small intestinal mucosa: 66% of intraluminal glutamine is metabolized in a single pass by rat jejunum \(^{112,113}\). Thus, most of the glutamine is metabolized in the small intestinal epithelium and does not reach the portal circulation \(^{17}\). Subsequently, it was shown that glutamine, and not glucose, is the main metabolic fuel of enterocytes. Extraction of glutamine from the circulation is directly related to blood glutamine level, and in conditions associated with a decreased circulatory glutamine level, such as starvation, glutamine utilization in the intestine declines, whereas, on the other hand, the hepatic glutamine uptake increases \(^{111}\).

In adult rats, 40% of the luminal arginine, absorbed by the intestinal mucosa, is catabolized in a single pass, and the remaining 60% enters the intestinal venous blood intact \(^{17,113}\). A similar percentage was found for humans \(^{114,115}\). These numbers indicate that substantial amounts of dietary arginine are not available for extra-intestinal tissues. For pig enterocytes, it was shown that 93, 4, and 1% of arginine was metabolized to ornithine, citrulline and CO\(_2\) respectively \(^{55}\). In adult pig enterocytes, the majority of the arginine that is metabolized to ornithine (56%), is subsequently metabolized to proline. However, in contrast to post-weaning pigs, proline is an essential amino acid in neonatal pigs \(^3\). This can be explained by the lack of arginase in the neonatal pig intestine.
### 4.2-Arginase I and II

#### 4.2.1-Tissue distribution

Arginase is the most widespread of urea cycle enzyme in terms of tissue distribution\(^{116}\). In mammals, the presence of at least two different arginase genes has been established\(^ {117-119}\). In man, hepatic arginase I (A-I) is primarily found in liver, but also in extra-hepatic tissues such as erythrocytes\(^ {120}\) and enterocytes\(^ {42}\). Studies in man and rats have demonstrated that liver and kidney-type arginase (A-II) differ from one another in both immunogenic and electrophoretic properties\(^ {121,122}\). Arginase II is more widespread and found primarily in kidney, adult intestine, stomach, brain, mammary gland, pancreas, epididymis, lung and skin\(^ {116}\). A-I and A-II may be coexpressed in the rodent kidney\(^ {123}\), small intestinal enterocytes\(^ {42}\), rat aortic endothelial cells and murine macrophages\(^ {116,124}\). The expression of A-II is highly species-dependent: the A-II content of small intestine and mammary gland of the rat is a factor 20 higher than that in man or dog\(^ {125,126}\).

#### 4.2.2-Enzyme properties

Ureotelic mammals excrete the ammonia that is formed during the catabolism of amino acids as urea. Arginine regulates urea synthesis by allosterically activating the synthesis of N-acetyl glutamate, the essential co-factor of CPS-1\(^ {127}\). The arginase reaction is highly exergonic with a change in free energy of -23 kcal/mol, making the reaction irreversible\(^ {128}\). Arginase has by far the highest specific activity of the ornithine cycle enzymes (a \(V_{\text{max}}\) of more than 4000 \(\mu\text{mol/min/mg}\)\(^ {128,129}\), but the high \(K_m\) of 1-15 mM causes it to work at much lower velocity in vivo. Its activity strongly depends on the presence of its cofactor Mn\(^ {2+}\)\(^ {130}\). Reflecting arginase's high capacity, tissue arginase activities and tissue arginine content are inversely related: liver, with the highest arginase content, has the lowest arginine content, while kidney, muscle and spleen have only 1% of liver arginase activity, but have a 5 to 10 fold higher arginine content (Fig. 3)\(^ {131}\). In addition to L-arginine, other substrates for arginase are: L-canavanine, L-
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homoarginine, L-argininic acid, and agmatine, but with 7-10 fold higher \( K_m \). D-arginine and GABA are not substrates \(^{129}\), indicating that the suitability of alternate substrates for liver arginase is determined by the presence of a guanidino group, the length of the amino acid carbon skeleton and the substituents around C-\( \alpha \). Lysine inhibits arginase \(^{132}\), and functions as an arginine antagonist in mammals \(^{133}\). As a result, the metabolic effects of feeding an arginine-deficient diet resemble the effect of feeding a diet containing a high lysine relative to arginine content.

4.2.3-Subcellular localization
A-I and A-II have a different subcellular localization: A-I is found in the cytosol \(^{134}\), though a substantial fraction of A-I (10 % of total activity) is associated with microsomes and mitochondria \(^{135}\). A-II is strictly associated with in the mitochondria \(^{136}\). It has been suggested that the mitochondrial localization of A-II facilitates the synthesis of glutamate and proline from ornithine \(^{116}\), given the intramitochondrial localization of ornithine aminotransferase.

4.2.4-Regulation of arginase expression
Activated murine macrophages metabolize arginine by two alternative pathways involving either the inducible enzyme NO synthase (iNOS) or arginase. Macrophages contain both A-I and A-II, with the A-II form predominating. Activation of macrophages, by LPS or \( \gamma \)IFN, induces a concurrent induction of iNOS and A-I \(^{137,138}\). In addition, arginine synthesizing enzymes ASS and ASL have been shown to be co-induced with iNOS \(^{139}\), implying that activated macrophages can regenerate arginine from citrulline and are independent of extracellular arginine for NO synthesis. However, cellular arginine import is elevated in macrophages when NOS expression is activated \(^{47}\), via enhanced expression of CAT-2 \(^{139}\), indicating that activated macrophages require extracellular arginine for sustained substrate supply for the generation of NO.
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Fig. 3: relation between arginase activity and arginine extractable content in several tissues. Adapted from Gopalakrishna, 1979. Open boxes: arginase activity; closed boxes: arginine content.

### 4.3-Agmatine

Agmatine is a metabolite of arginine, formed via arginine decarboxylase (ADC). Agmatine and ADC activity have been described to be widespread in brain, liver, kidney, intestine, adrenal glands and macrophages, but the function of agmatine is not well understood. Agmatine can be hydrolyzed by agmatine urohydrodrolase (agmatinase; EC 3.5.3.11) to putrescine, and hence agmatine is a metabolic precursor for the biosynthesis of higher polyamines. On the other hand, agmatine may suppress cell proliferation by inhibiting ODC activity and subsequent polyamine biosynthesis. In addition, agmatine may play a role in cell signaling by controlling NO production by inhibiting NO synthases. Recently, agmatine has been identified as an neurotransmitter in the brain, that binds the N-methyl-D-aspartate (NMDA) subclass of glutamate receptors in hippocampal neurons. Modulation of the receptor was shown to be mediated by the interaction between the guanidino group and the neuronal channel pore. Though
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the authors claimed this treat to be selective for agmatine \(^{145}\), other (neurotoxic) guanidino compounds (see Chapter 6), like guanidinosuccinic acid (GSA) and methylguanidine (MG) have been shown to share similar properties \(^{146,147}\).

4.4-Proline.

Ornithine can be converted by the enzyme ornithine aminotransferase (OAT) and proline oxidase via proline-5-carboxylate into proline. All these reactions occur in the mitochondrion \(^6\). Proline may stimulate collagen synthesis in hydroxylated form \(^{148}\). This may offer an explanation for arginine's ameliorating effect on the wound healing process and cell proliferation \(^{149}\).

4.5-Polyamines.

Ornithine also serves as precursor in polyamine synthesis and is decarboxylated by the rate-limiting enzyme ornithine decarboxylase (ODC; EC 4.1.1.17) to putrescine, the first polyamine in the biosynthetic pathway that can further be converted into spermine and spermidine. Polyamine synthesis has been correlated with increased arginase and ODC activity in the kidney \(^{150}\) and the small intestine \(^{151}\). Polyamines are known to exert regulatory properties in cell proliferation \(^{143,152}\) and, in that way, are involved in tissue growth and wound repair.

4.6-Nitric oxide.

Another, quantitatively minor, pathway in the urea cycle is the NO synthase (NOS) pathway in which the free-radical NO and citrulline are stoichiometrically formed by the action of NOS (EC 1.14.13.39) \(^{153}\). Three distinct isoforms of NOS are recognized, representing three different gene products \(^{154}\). Two of these isoenzymes, defined as cNOS, are expressed constitutively and are calcium-dependent, whereas one isoform, termed inducible NOS (iNOS or NOS II), is only synthesized \textit{de novo} after stimulation and is non-calcium dependent \(^{155,156}\). One cNOS subtype, named endothelial NOS (eNOS or NOS III), is mostly membrane-bound and is present in several cells, mostly endothelial cells \(^{157,158}\). The other
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cNOS subtype is cytosolic and mostly present in neuronal tissue and therefore defined as neuronal NOS (nNOS or NOS I). The iNOS isoform is cytosolic can be expressed in a great variety of cells including macrophages and epithelia. The cNOS enzyme is permanently present in cells and is (transiently) activated by temporary rises in free Ca\(^{2+}\). The iNOS isoform is not present at basal levels but can be induced in response to inflammatory cytokines and endotoxin, including LPS.
5- Regulation of metabolism by arginine

5.1 Effect of arginine on protein synthesis and degradation

During disease and injury the organism responds by changing the rates in the synthesis and/or degradation, i.e. turnover of proteins. Supplementation of intravenous or enteral arginine is associated with alterations in protein metabolism\(^{160}\). These effects may, at least in part, be mediated by the arginine-mediated hormone release such as insulin and growth hormone (GH), which are known to produce anabolic effects by inhibiting the loss of body protein and stimulating amino acid transport into the cells\(^{161}\) (see 5.2). The rate of translation of specific mRNAs determines the rate of protein synthesis, whereas post-translational modification of proteins affects protein function. Arginyl-tRNA is not only an immediate precursor for protein biosynthesis, but it is also involved in the post-translational conjugation of arginine with N-termini of proteins bearing N-terminal aspartate or glutamate, thereby targeting these proteins for degradation via the ubiquitin-dependent proteolytic pathway\(^{162}\). Arginine may thus be involved in modulation of protein breakdown by post-translational arginylation\(^{163}\).

5.2 Endocrine activity arginine

Arginine exerts a secretagogue effect by stimulating the secretion of the pituitary, pancreatic, and adrenal hormones. The pituitary GH has been shown to increase in response to intravenous administration of arginine in humans\(^{164,165}\), but also after oral administration\(^ {166,167}\). Arginine was also found to exert the release of prolactin, both after intravenous\(^{168}\) and oral\(^{167}\) administration. Furthermore, arginine administration stimulates the secretion of several pancreatic hormones like insulin\(^{169,170}\), glucagon\(^{171}\), pancreatic polypeptide\(^{172}\), and the GH-inhibiting hormone somatostatin\(^{173}\). This secretagogue effect of arginine has been shown to act via mechanisms dependent, as well as independent of the production of nitric oxide\(^{78,174}\). Several recent reports show that the effects of arginine constituting the induction of vasodilatation, inhibition of platelet
aggregation and blood viscosity are mediated, in part, by arginine-induced endogenous released insulin$^{175,176}$. Besides these pancreatic hormonal responses, arginine was also found to stimulate the secretion of catecholamines$^{175,177}$ by the adrenal gland.
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6-Guanidino compounds

6.1- the ornithine-guanidine bi-cycle

So far, approximately 120 naturally occurring guanidino compounds have been described in plants and tissues (for structural formulas: see Chapter VI, appendix II). Previously, Natelson and Sherwin proposed the existence of a guanidino cycle, which, next to the ornithine cycle, would be important in nitrogen metabolism. Though the intermediates have been detected in bacteria, the existence of this cycle has never been proven in mammals. This guanidine cycle was purported to be an alternative pathway to ensure the synthesis of creatine phosphate, needed for muscular contraction. In mammals, which do not possess urease, urea can be oxidized by the cytochrome P-450 system to give hydroxyurea. Hydroxyurea can either enter the ornithine cycle via the formation of carbamoylphosphate, or can be hydrolyzed to give hydroxylamine and enter the guanidine cycle. Guanidinoacetic acid (GAA), the precursor for creatine (CT), can be formed from glycine and arginine (via the ornithine cycle), or from glycine and canavanine (via the guanidine cycle). According to Natelson, the two cycles would be operative in parallel, and were therefore specified as the 'ornithine-guanidine bi-cycle' (Fig. 4). The existence of such a bi-cycle however, has never been shown in mammals.

6.2- guanidino compounds formed after transamidination.

A major route of formation of guanidino compounds from arginine is the transfer of the amidino group from arginine to glycine, yielding ornithine and GAA, the immediate precursor for creatine (CT) and creatinine (CTN). A proposed scheme of reactions is given in Fig 2. The reaction, catalyzed by the enzyme AGAT (EC 2.1.4.1), is reversible, and catalyzes the rate-determining step in creatine synthesis. AGAT accepts a wide variety of substrates as amidine acceptors: in addition to the physiological substrate α-amino acetic acid (glycine), α-aminopropionic acid (- α-alanine), α-aminobutyric acid or α-aminovaleric acid can
function as substrate, yielding guanidinopropionic acid (GPA), guanidinobutyric acid (GBA), and guanidinovaleric acid (GVA), respectively \(^{178,180,182}\) (Fig. 2).

Fig. 4: Proposed interaction of the ornithine cycle and the guanidine cycle via the “ornithine-guanidine bi-cycle” of Natelson et al. Excess urea can be re-utilized via this cycle. For details see text). Guanidine cycle substrates and products: hydroxyamine (OH-amine), homoserine (Hser), ureido homoserine (ureido-Hser), canavanino succinate (CavSA), canavanine (Cav), canaline (Ca). Intermediates in creatine synthesis: arginine (Arg), guanidinoacetate (GAA), creatine (CT), glycine (Gly).

In murine models of arginine deficiency, either as a result of OTC-deficiency (spf and spf-ash mice) \(^9^9\), or of A-I overexpression \(^4^2\), GPA, GBA, and GAA are decreased, demonstrating that availability of arginine as amidine donor determines the formation of these GCs.

6.3-formation of GSA by reactive oxygen species
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The formation of guanidino compounds under several pathological conditions is demonstrated, and some GCs, such as the neurotoxic guanidinosuccinic acid (GSA), guanidine (G), and methylguanidine (MG), have been reported to add to the (neuro)pathology of several metabolic diseases. Elevated levels of the guanidine derivatives MG as well as GSA, have been detected in the urine and plasma of patients with uremia, and both compounds were shown to be toxic. The exact mechanism of GSA synthesis remains to be established. Several alternative biochemical routes of GSA formation have been previously postulated. According to this proposed guanidine cycle pathway, GSA can be formed from canavaninosuccinate. As already stated, the existence of a guanidine cycle in mammals has never been demonstrated. Perhaps a more plausible route of GSA synthesis was proposed by Cohen et al.: GSA may be produced due to a shift in activity of AGAT to use aspartate rather than glycine as amidine donor. Another proposed enzymatic pathway of GSA synthesis is based on the cleavage of argininosuccinate by ASL to carbamoyl-aspartate and ornithine, instead of arginine and fumarate, and subsequent formation of GSA. This reaction may be stimulated by urea, which is known to inhibit ASL activity, thus favoring AS accumulation. Indeed, addition of urea to the medium dose-dependently increases GSA synthesis in isolated rat hepatocytes. In addition, urea levels correlate positively with GSA and MG concentration in patients with renal failure and after partial nephrectomy in rodents. On the other hand, the decreased GSA synthesis in human patients with hyperargininemia as a result of arginase deficiency cannot easily be explained by this hypothesis.

However, evidence is accumulating that the conversion of AS in GSA and betaine may be non-enzymatic, via the reaction of argininosuccinic acid with the action of an oxygen radical species. Such a conversion has already been shown to occur in vitro, in isolated rat hepatocytes. In a similar series of experiments, OTC inhibition by DL-norvaline inhibited GSA synthesis in isolated hepatocytes, suggesting that the urea cycle enzymes catalyze intermediate reactions in the GSA synthetic pathway. Following urea injection, hepatic GSA levels also increased...
in vivo, but there was little change in hepatic arginine\cite{190}, indicating that GSA synthesis is stimulated by urea, and is directly dependent on arginine availability. The latter is corroborated by the increased GSA synthesis in arginine-deficient transgenic mice\cite{42}.

Similarly, MG has been shown to be generated after the reaction of an oxygen radical with CTN\cite{191,192}. In uremia MG may arise from the degradation of CTN by the gut flora\cite{193}. The concentration of MG varies with the GSA concentration in patients with renal insufficiency. Together, the toxic GCs MG and GSA most probably are generated by the cleavage of CTN or AS respectively, by reactive oxygen. Such a mechanism would provide the rational for the use of active oxygen scavengers for treatment of uremia.

6.4-Pathological effects

The clinical syndrome of uremia is due to the failure of not only the excretory but also the metabolic, regulatory and endocrine functions of the kidney. In addition to elevations in CTN and urea, acute and chronic renal failure is recognized by a state of hypercitrullinemia\cite{194}. Citrulline proved to be a more sensitive indicator than urea or creatine, as citrulline levels correlated with the degree of nephrectomy, and hypercitrullinemia developed in the range of 10\% to 33\% nephrectomy without any changes in urea and creatine\cite{194}. Thus, hypercitrullinemia is a specific marker of normal function of the proximal tubule, whereas an impaired creatine and urea clearance apparently can be compensated. A number of guanidino derivatives are also considered to be important indicators of renal failure\cite{193}. GSA positively correlates with plasma urea\cite{186} and indicates the severity of renal failure\cite{195,196}. MG also appears in plasma after renal failure\cite{197}. GSA and MG are recognized as candidate markers, which reflect the pathological stage of nephritis\cite{147,198,199}. The enhanced production of certain GCs, in particular GSA and MG, has been associated with the etiology of the uremic syndrome. Their increased synthesis has been reported in renal insufficiency\cite{184,196,200,201}, in partially nephrectomized rats and mice\cite{187}, during endotoxemia-induced sepsis\cite{202},
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and in patients with hypothyroidism\textsuperscript{203}. Furthermore, an increased GSA concentration is found during under hyperammonemic conditions due to consumption of an arginine-free diet in carnivorous species\textsuperscript{204}, or under normal ammonia concentrations in mice models of arginine deficiency\textsuperscript{42}.

Uremia has been shown to be associated with neurological disorders\textsuperscript{205-207}, and a proportion of patients with renal failure suffers from central and peripheral nervous system impairments\textsuperscript{206}. Mildly uremic mice display acute behavioral deficits, which may relate to analogous symptoms in uremic patients suffering from uremic encephalopathy\textsuperscript{208}. The role of GSA in uremic neurotoxicity and coma is still controversial and needs further investigation, but GSA, G, and MG have been shown to be (neuro)toxic \textit{in vitro}\textsuperscript{209}. Moreover, GSA has been shown to cause convulsions after intracerebroventricular injection in mice\textsuperscript{147}. The elevated levels of GSA and MG may well play a role in the neurological consequences of uremia via activation of NMDA receptors\textsuperscript{147,210}. Mice, suffering from low levels of arginine and elevated level of circulating GSA and MG displayed behavioral deficits, such as hyperactivity as well as several more specific deficits in neuromotor abilities and coordinated movements\textsuperscript{42,98}.
Introduction

7-Arginine transporters

7.1-Amino acid transporters

Five systems are known to mediate transport of cationic amino acids over the cell membrane: system $y^+$, $y^+L$, $b^+$, $b^{0}$, and $B^{0}$. These transport systems can be distinguished by their affinity for cationic amino acids, their dependence on sodium, and their capacity to share transport with zwitterionic amino acids. Systems $b^+$, $b^{0}$, and $B^{0}$, first discovered in rat blastocysts, are found in brush border membranes, and have a broad specificity for zwitterionic and dibasic amino acids. Systems $b^+$ (cationic amino acids-preferring) and $b^{0}$ (cationic and neutral amino acids-preferring) are sodium-independent, and $B^{0}$ (cationic and neutral amino acids-preferring) is sodium-dependent. Transport of arginine, ornithine, and lysine across plasma membranes is mostly performed via the sodium-independent amino acid-transport system, formerly designated $y^+$.

7.2-System $y^+$

The major mammalian cationic amino acid-transport system is the nearly ubiquitous system $y^+$, which facilitates Na$^+$-independent arginine, lysine, and ornithine transport driven by membrane potential. Expression in oocytes revealed the ecotropic murine leukemia virus receptor, now named CAT-1, as a CAT. Since, four homologous human and rodent genes make up the family of cationic amino acid transporters (CATs) that encode system $y^+$, CAT-1, 2A, and a splice variant CAT-2B, CAT-3 and CAT-4.

CAT-1 expression is nearly ubiquitous and produces a single protein. Under basal conditions, CAT-1 is absent from hepatocytes, probably to prevent unnecessary transport and metabolism of arginine by the hepatic arginase in the hepatocytes. However, CAT1 expression is present in regenerating liver, and in the cases when hepatic cationic amino acid transport is needed, such as following feeding, cellular growth and illness.
A full length cDNA clone from a previously identified murine T-lymphoma cell line (Tea, T early activation gene) showed significant homology (61%, \(218\)) with CAT-1 and amino acid-transport capacity in oocytes \(219\). Two splice variants of this gene were identified and termed CAT-2A and -B. mCAT-2 expression is highly tissue-specific and is expressed using at least two widely separated promoters \(51\). CAT-2A is expressed in the liver and is regarded as the low affinity variant, whereas CAT-2B is the high affinity variant, expressed in T-cells. CAT2A is highly expressed in liver, skeletal muscle, especially after trauma, and skin, while CAT2B expression is high in brain, lung, testis, uterus, and activated, but not resting T-cells (expression was found in T-cell thymoma cells, but was low in thymocytes \(215\)). CAT 2A and 2B proteins are 97% identical, as they differ only one 41 amino acid domain \(215,219\).

Recently, two new members of the CAT family have been cloned; a brain-specific isoform, which again has strong similarity with CAT-1, termed CAT-3 \(220\), and CAT4 (previously termed HCAT3). The latter gene is highly expressed in skeletal muscle, intestine, kidney, and placenta and in eye and in retinal pigmented epithelium. CAT4 is homologous to the amino acid permease CD98 light chain \(221\). A defect in CAT4 expression at the basolateral membrane of epithelial cells in kidney and intestine has been associated with the development of lysinuric protein intolerance, a rare autosomal recessive disease, characterized by poor feeding, vomiting, diarrhea, and episodes of hyperammonemic coma \(222,223\).

### 7.3-Regulation of arginine transporters

CAT1 and CAT2 proteins expressed in Xenopus oocytes exhibit high-affinity-low capacity cationic amino acid transport activities \(224\). There is a steric constraint for arginine binding to the CAT-1 transporter as \(\alpha\)-methylation completely abrogates arginine binding \(217\). Similarly, the affinity for D-arginine is 20 times less than for the L-arginine \(225\). On the contrary, L-homoarginine is specifically transported with high affinity, making it a useful probe to study CAT-1 activity, as physiological concentrations of L-homoarginine are low. The kinetic
constants $V_{\text{max}}$ (maximal transport rate when excess substrate is present) and $K_m$ (substrate concentration at which the transport rate is half maximal) for arginine uptake depend on the CAT isoform. In hepatocytes in the basal state, the $y^+$ system (CAT2A) is barely detectable having a $K_m$ value of 2-5 mM and a $V_{\text{max}}$ of 3.3 nmol $^{-1} \cdot$ mg of protein $^{-1} \cdot$ min$^{-1}$ i.e. a 10-70 fold higher apparent $K_m$ and $V_{\text{max}}$ than the CAT1 and 2B (100-150 µM). The high $K_m$ value of CAT2A for arginine (way above the normal plasma concentration) underscores the minimal rate of arginine uptake by hepatocytes under normal physiological conditions. The precise cellular localization of arginine transporters may account for the "arginine paradox", the dependence of endothelial NO synthesis on extracellular arginine availability, while the intracellular arginine concentrations (0.1-1.0 mM) greatly exceed the $K_m$ of eNOS for arginine (2-10 µM). The apparent $K_m$ for arginine of NO synthesis by intact endothelial cells is approximately 75-150 µM, which is in the range of normal physiological plasma concentrations, and of the $K_m$ values of the arginine transport systems.

Glucocorticoids and insulin induce expression of the CAT1 gene in liver cells through induction of transcription and stabilization of its mRNA. The CAT1 gene is subject to adaptive regulation by arginine availability in vitro, as arginine depletion increases CAT1 mRNA stability, causing an increase in CAT1 protein, and transport capacity. CAT2A is, in contrast to CAT1 and 2B, hardly trans-stimulated. Therefore, the 41 amino acid domain which lacks in the protein sequence of CAT2A is thought to be responsible for substrate recognition and possibly the mechanism of substrate translocation.

Quiescent lymphocytes from spleen, lymph nodes, and Peyer's patches constitutively express CAT2B, although these cells exhibit little transport of arginine via the systems $y^+$ or $y^+L$. Upon mitogenic activation, however, transcripts of CAT2B rapidly accumulate, underscoring a role for arginine in this T-cell activation process. In addition, system $y^+$ can be induced by inflammatory cytokines in hepatocytes and macrophages.
Other cationic amino acids and positively charged analogues are effective inhibitors of arginine uptake by system \(y^+\). For example, arginine uptake can be competitively inhibited by lysine, ornithine, canavanine, and certain NOS inhibitors, such as N-monomethyl-L-arginine and N-iminoethyl-L-ornithine. N-nitro-L-arginine and N-nitro-L-arginine methyl ester, or aminoguanidine, on the other hand, have no effect on arginine uptake.
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8-Arginine as immunonutrient.

8.1 Arginine and immune function

Only limited information is presently available as to how nutrients can influence immune function or the development of lymphoid organs. An intriguing feature of the nutritional benefit of arginine is, next to its function as a building block for protein synthesis, its immunosupportive effect, especially under catabolic conditions. Arginine was already identified as an immunonutrient more than 20 years ago, in studies on wound healing. Since, arginine has often been indicated to influence immune system responses, either via the increased production of polyamines, effects on tumor growth, by ameliorating survival during sepsis, or directly on T-cell gene expression. Based on these studies, arginine has widely been added to postoperative supplemental formulas, like Impact® (Novartis), at doses as high as 100 gr per kg formula, but the actual molecular basis of the beneficial effect of arginine on lymphocyte biology has remained unclear through the years.

8.2 Potential mechanism of action

ADP-ribosylation of arginine residues

In the transgenic arginine-deficient mice described in Chapter V, the development of the lymphoid system is affected. This finding may hint towards an important aspect of arginine's immunosupportive effect. The influence of the arginine deficiency seems to be restricted mainly to the early development of B-cells in the bone marrow, as we found no defective T-cell maturation in the thymus, or reduction of T-cell number in peripheral lymphoid organs. Despite their hampered maturation, transgenic B-cells residing in the spleen were able to proliferate normally upon B-cell specific in vitro stimulation. We are currently testing the proliferative ability of splenic T cells, but preliminary data already

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indicate an impaired production of IFNγ, and possibly IL4 and 6, upon CD3/CD28 in vitro stimulation, corroborating other reports\(^\text{228}\).

In addition to a temporary hampered B cell maturation, the F/A transgenic mice suffer from a temporary inhibition of muscle development. An attractive hypothesis on how arginine may influence both lymphocyte responses and muscle growth is a possible enhancement of ADP-ribosylation of arginine residues in lymphocyte membrane proteins. Mono-ADP-ribosylation is a posttranslational modification of proteins in which the ADP-ribose moiety of NAD\(^+\) is transferred to the guanidino group of arginine on a target molecule\(^\text{241}\). This reaction is catalyzed by mono-ADP ribosyltransferases (ART), which are exo-enzymes imbedded in the cell membrane. ART is abundantly expressed in myocytes, as well as immune cells. A membrane protein that was identified as a substrate for ART was \(\alpha7\)-integrin\(^\text{242}\). Mice, lacking this integrin suffer from muscular dystrophy, which starts five days after birth\(^\text{243}\), a phenotype similar to the arginine-deficient F/A-mice.

The involvement of ART in immune response regulation is already indicated by the observation that strains of rats, which lack one of the ART enzymes (ART-1), develop autoimmunity\(^\text{244}\). ART-1 and 2, isoforms of the murine enzyme, which are active on CD4\(^+\) and CD8\(^+\) T-lymphocytes, and may be functional in downregulating their activation state\(^\text{245}\). ART-1 and 2 have been shown to specifically ribosylate members of the integrin family of adhesion molecules, like lymphocyte-function associated protein-1 (LFA-1)\(^\text{246}\), or T-cell membrane proteins important in eliciting T-cell mediated immune responses, like CD27, CD28, CD43, CD44 and CD45R\(^\text{242}\). ADP-ribosylation of T-lymphocytes is known to attenuate T-cell proliferation, cytotoxicity, and cytokine excretion\(^\text{247}\), probably because T-cell receptors fail to associate in a contiguous and functional receptor cluster after stimulation\(^\text{242}\). B-cell responses are not affected by ADP-ribosylation\(^\text{245}\). Hence, the observed inactivity of T-lymphocytes (preliminary data), but intact B-cell response upon stimulation in our F/A-2 transgenic mice perfectly matches the functional consequences of an over-ribosylation of T-cells. Such an over-ribosylation during hypo-argininemia could be due to the fact that ARTs not only
ADP-ribosylate arginines in the backbone of proteins, but also free arginine. Thus, arginine and agmatine are the naturally competitive inhibitors of ADP-ribosylation of cell-surface molecules, and a decrease in circulating arginine, as seen in our F/A transgenics, then results in an enhanced ribosylation of cell surface proteins. However, the involvement of ADP-ribosylation in the arginine deficiency phenotype remains to be demonstrated.
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9-Scope of the thesis

The amino acid arginine is more than a building block for the synthesis of protein; it serves important physiological functions, such as the detoxification of ammonia, and it is a precursor for the synthesis of nitric oxide (Science’s molecule of the year in 1990), agmatine, creatine and polyamines. Furthermore, it serves as a secretagogue for glucagon and growth hormone, and evidence is accumulating that it has intriguing capacities in promoting lymphocyte function. Lactating mammals have been shown to accumulate large amounts of arginine, compared to mother’s milk, implying endogenous arginine biosynthetic capacity. This thesis aims to establish the specific functions of arginine for neonatal development.

In Chapter II, we identified the suckling small intestine as an organ with arginine biosynthetic capacity, after studying the spatial distribution of ornithine cycle enzymes along the rat small intestinal villus axis. The enterocytes of neonatal and suckling rats have the capacity to synthesize arginine, but this capacity is shown to be lost after weaning. Furthermore, we found a compartmentation of the citrulline and arginine synthesis over the villus axis: the basal enterocytes seem to synthesize citrulline, and the apical enterocytes arginine. A possible role of the changes in turnover rate of enterocytes during development in the establishment of this metabolic compartmentation is discussed.

We investigated the significance of this intestinal arginine metabolism for neonatal development further in a murine model of chronic arginine deficiency. To this end, two lines of transgenic mice which express different levels of arginase I in their enterocytes were generated, in order to establish lines of mice with a graded severity of arginine deficiency. In Chapter III, both lines were analyzed, and indeed this approach led to a, surprisingly selective, reduction in circulating arginine concentration, further underscoring the importance of enterocytes in maintaining arginine homeostasis. We describe the development of a pronounced phenotype in the arginine-deficient mice, which corresponds with the degree of arginine
deficiency and which includes the retardation of hair- and muscle growth, and an impaired development of the lymphoid tissue, in particular Peyer’s patches.

As mentioned earlier, arginine serves as a precursor for the synthesis of creatine and other guanidino compounds. In Chapter IV, the metabolic effects of a deficiency of arginine, in particular of arginine’s guanidino group, are considered. Decreases in the concentration of arginine lead to corresponding decreases in its transaminidation products and, hence, creatine synthesis. However, hypoargininemic conditions also induce the accumulation of certain neurotoxic guanidino compounds, associated with the development of psychomotor deficits, a phenomenon which parallels the enigmatic guanidino compound disturbances seen in patients with renal failure or hyperammonia. These data hint to a role for an enhanced oxidative stress under these conditions.

Another feature of arginine is its purported immunosupportive effect, which has never been explained, despite more than 20 years of research on this matter. As described in Chapter III, hypoargininemia affects the development of lymphoid organs, reflected in the virtual absence of Peyer’s patches. In Chapter V, this feature is further explored. Arginine deficiency leads to reduced B-cell numbers in the peripheral lymphoid organs, but T cells numbers are unaffected. Further research into the cause of this reduction led to the bone marrow, where it was unexpectedly revealed that arginine deficiency impaired early B-cell maturation at the transition from the pro- to pre-B cell stage
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Arginine-metabolizing enzymes in the developing rat small intestine

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Summary

Prior to weaning, arginine biosynthesis from citrulline most likely takes place in the small intestine rather than in the kidney. We studied the expression of ornithine cycle enzymes in the rat small intestine during perinatal development. The spatio-temporal patterns of expression of ornithine aminotransferase (OAT), carbamoylphosphate synthetase (CPS), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG) mRNAs were studied by Northern-blot analysis and in situ hybridization. In addition, the expression of carbamoylphosphate synthetase and argininosuccinate synthetase protein was studied by immunohistochemistry. Before birth, the developmentally more mature proximal loops of the intestine expressed the mRNAs at higher concentrations than the more distal loops. After birth, this difference was no longer obvious. The mRNAs of argininosuccinate synthetase and argininosuccinate lyase, the enzymes that metabolize citrulline to arginine, were only detectable in the upper part of the villi, whereas the other mRNAs were concentrated in the crypts. The distribution of argininosuccinate synthetase protein corresponded with that of the mRNA, whereas carbamoylphosphate synthetase protein was present in all enterocytes of the crypts and villi. Hepatic arginase mRNA could not be detected in the enterocytes. The spatial distribution of the respective mRNAs and proteins along the villus axis of the suckling small intestine indicates that the basal enterocytes synthesize citrulline, whereas the enterocytes in the upper half of the villus synthesize arginine.
Ornithine-cycle enzymes in the rat gut

Abbreviations:
ARG [EC 3.5.3.1] : Arginase
ASL [EC 4.3.2.1] : Argininosuccinate lyase
ASS [EC 6.3.4.5] : Argininosuccinate synthetase
CPS [EC 6.3.4.16] : Carbamoylphosphate synthetase
OAT [EC 2.6.1.13] : Ornithine aminotransferase
OTC [EC 2.1.3.3] : Ornithine transcarbamoylase
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**Introduction**

Arginine, an intermediate metabolite of the ornithine cycle, is considered to be a dispensable amino acid for most healthy adult animals [1]. Renal arginine biosynthesis, the main source of endogenous arginine in the adult [2], does not vary with fluctuations in dietary arginine intake [3-5], but depends on the vascular supply of its precursor citrulline [6]. Intestinal citrulline synthesis from glutamine is, in turn, the main source of circulating citrulline [7,8]. The liver is not considered to contribute to circulating citrulline or arginine levels because of its high levels of ASS, ASL and ARG. In contrast to the situation in adults, arginine is regarded as a semi-essential nutritional compound in many adolescent animals, including rats, because it is synthesized by the kidney at rates that are inadequate to support rapid growth [9]. This discrepancy between the requirement for arginine and its endogenous biosynthetic capacity may even be aggravated in suckling rats, since the enzymes that convert citrulline to arginine in the kidney only rise to adult levels towards weaning [10,11]. Nevertheless, the finding that the ratio of the amino acid composition of rat body and milk is approximately 1 for essential amino acids, whereas it is more than 2 for arginine (and glycine) [12], clearly suggests a high degree of endogenous arginine biosynthesis during the suckling period.

The ornithine cycle enzymes except arginase are, expressed in the small intestine of suckling rats [7,10,13,14], but data on their cellular distribution are not yet available. By analysing the expression and localisation of ornithine-cycle enzyme mRNAs and protein by Northern blotting, *in situ* hybridization, and immunohistochemistry, we provide evidence that newborn rats have the capacity to synthesize citrulline and arginine at the base and the upper part of the villus of the small intestine, respectively.
Materials and Methods

*Animals.* Adult Wistar rats were obtained from the HSD animal farm in Zeist (The Netherlands). Timed matings were used for the study of fetal animals. The day of copulation was taken as day 0 of pregnancy (ED 0). Birth normally occurred at the beginning of day 22 of pregnancy. The animals were weaned at three weeks of age.

*Preparation of tissue sections.* Serial tissue sections were prepared exactly as described previously [15]. Transverse sections in the abdominal region of prenatal animals were used, while of postnatal animals the proximal part of the jejunum, approximately 5 cm distal to the ligament of Treitz, was dissected free and processed further.

cRNA probes. The following cDNA fragments were cloned into the pBluescript vector to generate cRNA probes for the detection of the different ornithine cycle mRNAs: carbamoylphosphate synthetase I (CPS), the 564 bp BamHI-SmaI fragment of the rat cDNA clone pBR-CPS5 [16]; ornithine transcarbamoylase (OTC), the 870 bp XbaI-HindIII fragment from the rat cDNA clone pOTCl [17]; argininosuccinate synthetase (ASS), the 1300 bp PstI fragment from the rat cDNA clone rAS [18]; argininosuccinate lyase (ASL), the 580 bp EcoRI-HindIII fragment from the rat cDNA clone pALr-3 [19]; arginase (ARG), the 768 bp PstI-StuI fragment from the rat liver cDNA clone pARGr-2 [20,21]; ornithine aminotransferase (OAT), the 1850 bp from the OAT cDNA clone pRLOT10 [22]. Probes were prepared by in vitro transcription of the appropriate DNA strand and had a specific activity of $1.5 \times 10^9$ cpm/µg. Onto each section 10 µL probe containing 40,000 cpm per µL was applied.

*In situ hybridization.* A comprehensive protocol to detect mRNA molecules in tissue sections using radioactively labelled cDNA probes has been described.
previously [15]. At variance with this protocol the temperature of hybridization and washes was raised to 55 °C as cRNA rather than cDNA was used.

_Northern-Blot Analysis._ Total RNA was isolated as described [23]. Ten micrograms of total RNA, denatured by heating for 10 minutes at 65 °C in the presence of 2.2 M formaldehyde, was electrophoresed on an agarose gel, containing formaldehyde. After transfer onto a nylon membrane (Amersham International plc., Little Chalfont, Buckinghamshire, U.K.), the RNA was hybridized to [$\alpha^{32}$P]-cytidine triphosphate (CTP)-labelled (Amersham) cDNA probes derived from the same clones used for the cRNA labelling in the _in situ_ hybridizations. Hybridization was performed in 50% formamide, 5 times Denhart's solution (0.1 % Ficoll 400, 0.1 % polyvinylpyrrolidone, 0.1 % bovine serum albumin (fraction V)), 0.5% SDS and 5 times SSC (0.75 M NaCl, 75 mM Na$_3$Citrate). cDNA probes were labelled using the random primed labelling method. After washing, the blots were exposed to phosphor screens for approximately 15 hs, and signals were quantified using the Phosphorimager software (Molecular Dynamics, Sunny Vale, CA).

_Immunohistochemistry._ After deparaffination and rehydration, endogenous peroxidase activity in the sections was eliminated by incubation for 30 min in PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH=7.4) and 50 % methanol, containing 3 % (wt/vol) hydrogen peroxide. Non-specific protein binding sites were blocked by incubation for 30 min in TENG-T buffer (10mM Tris, 5 mM EDTA, 150 mM sodium chloride, 0.25 % gelatin, 0.05 % Tween-20, pH=8.0. Serial sections were incubated overnight with an appropriate dilution of rabbit polyclonal antibodies against CPS [24], and ASS [25,26]. The indirect unconjugated peroxidase-anti-peroxidase technique [27] was used to visualise binding of the primary antibodies, with 3,3-diaminobenzidine (0.5 mg/ml) as a substrate, dissolved in imidazole buffer (30 mM imidazole, 1 mM EDTA, pH 7.0), to which 0.01 % hydrogen peroxide was added.
Arginase activity measurements. Arginase activity was measured by a modification of the assay described by Adlung et al [28]. Fifty mg of tissue was homogenised in 2 mL of a 30 mM sucrose buffer containing 5 mM MnCl₂, 30 mM imidazole (pH 7.5), and 0.05 % Triton X-100. This homogenate was sonicated for 30 s and activated at 55 °C for 15 min. The mixture was diluted with 1 vol H₂O and centrifuged. The assay mixture (200 μL) contained 20 μL of the enzyme extract, 25 mM (guanido-¹⁴C)-labelled L-arginine (pH 9.5, 1.0 Ci.mol⁻¹; Dupont NEN), and 75 mM glycine (pH 9.5). The reaction was stopped after 6 min of incubation at 25 °C by addition of 100 μL 0.5 M L-arginine (pH 9.5) and 800 μL 30% (wt/vol) phosphotungstic acid (P₂O₅; Malinckrodt Baker, Deventer, The Netherlands). After centrifugation, 100 μL of the supernatant was counted.
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Results

Developmental changes in mRNAs. Figure 1 shows the developmental changes of CPS, OTC, ASS, ASL and OAT mRNA levels in the small intestine of the rat. The levels are expressed per μg of total RNA and relate to the corresponding mRNA levels in the adult liver, demonstrating that all mRNAs except ASL were highly expressed in the suckling small intestine. mRNA levels of all genes were highest during the suckling period. ASS and ASL mRNAs declined to hardly detectable levels in the second postnatal week, whereas mRNA levels of CPS and OAT declined to adult levels in the third postnatal week. OTC mRNA level did not change with development. However, it should be acknowledged that the mRNA levels were determined per μg of total RNA, so that our data do not take into account the increase in epithelial surface as a result of the growth of the intestine. The mRNA concentration of CPS and OAT was approximately twofold higher in the ileum than in the jejunum, whereas concentrations of the other mRNAs studied were approximately equal in both parts of the small intestine. After birth, the horizontal gradient was either no longer detectable (OTC, ASS, ASL), or had even become reversed, with the highest concentration in the distal (ileal) parts of the small intestine (Figure 1, CPS and OAT).

The cellular distribution of the mRNAs pre- and postnatally was investigated using in situ hybridization. The earliest stage investigated was ED 16. Even though the small intestine is still immature at this age, the enterocytes expressed CPS, GDH, ASS and OAT mRNAs, but not yet OTC and ASL (not shown). Two days later in development (Figure 2), the presence of intestinal villi could be observed in the proximal loops of the small intestine, but not yet in the distal ones, in line with the cranio-caudal gradient of development. The expression of CPS, OTC, ASS and OAT mRNAs was stronger in the more mature, proximal part of the small intestine, compared to the distal part, as can be seen from the difference between loops where intestinal villi are present and loops with a simple epithelial lining of the lumen (Figure 2). While the morphogenesis of the villi continued towards birth, the expression of CPS, OTC, ASS, and OAT mRNAs
remained essentially unchanged (shown for ASS and CPS, Figure 3). ASL mRNA was undetectable prenatally.

Fig 1. Developmental appearance of mRNAs in the male rat jejunum (triangles) and ileum (rectangles), as determined by Northern-blot analysis. The expression of OAT (panel a), CPS (panel b), OTC (panel c), ASS (panel d), ASL (panel e) was measured by quantification of the hybridization signal using the Phosphorimager (Molecular Dynamics). mRNA levels are expressed as a percentage of the signal in a reference sample of adult rat liver RNA. On the X-axis, the age is given in neonatal days after birth. Five animals were analysed per age group. Values are means ±SEM.
Figure 2. Expression of the mRNAs encoding CPS (a), OTC (b), ASS (c), ASL (d), ARG (e) and OAT (f) in transverse serial sections, showing the abdominal part of an ED18 rat fetus. The difference in development and expression of tissue-specific mRNAs between the proximal (pi) and distal (di) intestinal loops is very pronounced. Liver (li). Bar: 250 μm.

After birth, the developmental changes in the intensity of the hybridization signals in the proximal part of the small intestine (Figures 4, 5), reflected that of the Northern blots: the expression of CPS, ASS, ASL and OAT was highest at 7 days,
and had clearly declined at 17 days, whereas the expression of OTC remained fairly constant.

Figure 3. Expression of the mRNAs encoding CPS (a) and ASS (b) in transverse serial sections of an ED20 rat fetus, showing liver (li) and proximal (pi) and distal (di) intestinal loops. Note that the expression of CPS mRNA is distributed along the entire villus, including its base, whereas ASS mRNA is absent from the villus-base. ASS mRNA is expressed in the enteric neurons of the myenteric plexus (arrowhead). Only very weak hybridization was seen in the distal parts of the intestine. Bar 100 μm.

In the adult jejunum CPS, OTC and OAT mRNAs were still expressed, but the expression of ASS and ASL mRNAs was no longer detectable, except in the neurons of the myenteric plexus (Figure 6). GDH mRNA continued to be expressed in the adult small intestine (not shown).

Zonation of expression along the crypt-villus axis. Heterogeneity along the axis of the villus evolved parallel with villus development. At ED 18 the expression of
CPS, OTC and OAT mRNAs was seen in the epithelium along the entire villus (Figure 2), whereas ASS levels were weaker at the intervillus region than at the upper part of the villi.

Figure 4. Expression of CPS (a), OTC (b), ASS (c), ASL (d), ARG (e) and OAT (f) mRNAs in serial sections of the jejunum of 7 day-old neonatal rats (ND7). The difference in distribution along the villus of CPS, OTC and OAT mRNAs on the one hand and ASS and ASL mRNA on the other is very pronounced. Note the silver grains in panel e, which were found not to be due to the presence of ARG mRNA (see text). Bar: 100 μm.
Figure 5. Expression of CPS (a), OTC (b), ASS (c), ASL (d), ARG (e) and OAT (f) mRNAs in serial sections of the jejunum at ND17 of rat development. Note the reduced expression of ASS and ASL mRNA compared to Figure 3. Bar: 100 μm.
Figure 6. Expression of CPS (a), OTC (b), ASS (c), ASL (d), ARG (e) and OAT (f) mRNAs in serial sections of the jejunum of an adult rat. CPS, OTC and OAT mRNAs have become confined to the crypts and lower part of the villus. Note the expression of ASS mRNA in the neurons in the myenteric plexus. Bar: 250 μm.

Figure 7. Presence of CPS (a, c, e, g, i) and ASS (b, d, f, h, j) protein in the rat jejunum of ED 20 (a, b), ND 1 (c, d), ND 8 (e, f), ND 18 (g, h) and adult (i, j). ASS protein is only present at the upper half of the villi, whereas CPS protein is seen in the developing crypts and the enterocytes along the entire villus. In the adult jejunum, ASS protein is only detected in the enteric neurons (arrowheads). Bar: 100 μm.
At ED 20 the concentration of CPS, OTC and OAT mRNA was higher at the intervillus region, whereas ASS mRNA was absent from this part of the epithelium (shown for CPS and ASS, Figure 3). After birth, this difference became more pronounced: ASS and ASL were only detectable in the upper part of the villi, whereas CPS, OTC and OAT were present mainly in the basal part of the villi and in the developing crypts (Figures 3-5).

Antibodies against two key enzymes, CPS and ASS were applied to investigate to what extent mRNA and protein colocalize (Figure 7). Whereas CPS mRNA was concentrated at the basal part of the villi and in the developing crypts, CPS protein was present in all enterocytes. ASS protein, on the other hand, colocalized with ASS mRNA, and was confined to the upper part of the villi. ASS protein was, except for the enteric neurons, no longer detectable in the adult intestine, while CPS protein remained present.

Arginase. In order to demonstrate that the small intestine of the suckling rat indeed has the capacity to synthesize arginine, arginase activity should be absent. Arginase mRNA was undetectable at all ages investigated, both by in situ hybridization and Northern-blot analysis. The only exception was a highly reproducible in situ hybridization signal at ND 7 (Figure 4, panel e). This signal could not be eliminated by RNAase treatment of the section before hybridization (not shown).

Moreover, arginase mRNA was not detectable on Northern blots (Figure 8, panel a) and arginase activity was virtually undetectable in jejunal extracts before ND 18 (Figure 8, panel b). Therefore, the in situ hybridization signal observed at ND7 was judged to be non-specific. After ND18, arginase activity in the jejunum showed an abrupt increase. The adult values were found to be $8.7 \pm 0.5 \%$ (n=5) of the activity measured in the adult rat liver.
Ornithine-cycle enzymes in the rat gut

A  Hepatic arginase in the jejunum

B

Figure 8 panel a. Northern-blot analysis showing the absence of hepatic arginase mRNA in rat jejunum. The blot is representative for five separate experiments, run in parallel with those shown in Figure 1, panels a-e. Panel b. Development of arginase activity in homogenates of rat jejunum. On the X-axis the age of rats is given, on the Y-axis the enzyme activity in nmol/min/mg protein. Values are means ± SEM; n = 5 animals per age group.
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Discussion

The metabolic demands and capacities of organs can change during development. An intriguing example is found in the small intestine. In adult mammals, the intestine produces citrulline from glutamine. After release of citrulline into the circulation, the kidney converts citrulline into arginine. However, the newborn kidney acquires the capacity to synthesize arginine from citrulline only gradually, with adult levels being established around weaning [11,29]. Prior to weaning, arginine biosynthesis most likely takes place in the gut rather than the kidney [10], because the activities of the enzymes involved in arginine biosynthesis are high, and those that utilize arginine or its precursors are low.

In the small intestine of mice, rats, and pigs, the activities of the enzymes involved in citrulline biosynthesis (CPS, N-acetylglutamate synthetase (AGAS) and OTC), as well as those involved in the conversion of citrulline into arginine (ASS and ASL) peak shortly after birth and gradually decline to adult levels at weaning [10,30-33]. Similarly, enzymes that convert glutamine to ornithine (phosphate-dependent glutaminase (PDG), pyrroline-5-carboxylate synthase (P5CS) and OAT all show activities well above adult levels in the period before weaning [13,14,31,33-36]. Since neonatal transgenic mice, in which the OAT gene was disrupted, have very low circulating arginine levels, it is plausible that the activity of OAT in the small intestine is directed towards the synthesis of ornithine in the neonatal period [37]. On the other hand, the activity of pyrroline-5-carboxylate reductase (P5CR), the enzyme that can divert pyrroline-5-carboxylate towards proline, is low during the suckling period in rat and hamster [35,38]. Furthermore, ornithine decarboxylase (ODC) activity, which diverts ornithine towards polyamine biosynthesis, is very low during the suckling period, but shows a sharp, transient burst in activity at the onset of weaning [14,39]. Finally, we and others [10,14,38,40] found that intestinal arginase activity is virtually absent in the intestine until it appears abruptly at weaning.
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The absence of a hybridization signal with our ARG probe in gut tissue at a time when arginase activity is easily measurable, i.e. after weaning, shows that this arginase activity arises from an arginase isoform that is different from that in liver [41-43]. Indeed, an immunologically distinct arginase in rat mammary gland and kidney has been isolated [44], and cloned [45,46]. The cellular distribution of this non-hepatic arginase gene-product in the gut remains to be established.

The ASS mRNA and protein in the neurons of the myenteric plexus of the adult small intestine colocalise with neuronal nitric oxide synthase mRNA (unpublished results), suggesting the existence of a neuronal arginine-citrulline cycle for regeneration of arginine for nitric oxide synthesis. Such a cycle has been described in several other nitric oxide producing cell-types, like endothelial cells [47,48] and macrophages [49].

Perhaps the most intriguing aspect of the present study is why the mRNAs for glutaminase [13,50], CPS, OTC and OAT mRNAs are concentrated at the base of the villi and in the developing crypts, whereas ASS and ASL can only be demonstrated in the upper part of the villi. This difference in distribution can already be detected at ED 18, is easily visible at ED 20, and persists until weaning. The question arises in what respect the enterocytes occupying the upper half of the villus in the fetal and neonatal intestine, differ from those occupying the same position after weaning. In this respect, it might be of relevance that dramatic maturational changes occur in the rat small intestine in the third postnatal week, including an acceleration of the turnover rate of enterocytes [51]. It is tempting to relate the disappearance of ASS and ASL gene-expression from the enterocytes in the third week to this higher turnover rate of the enterocytes. ASS and ASL can apparently only be expressed in the enterocytes with a long lifetime. Another conspicuous property of suckling enterocytes is the presence of a giant supranuclear lysosome. These highly typical cells disappear at weaning and, prematurely, upon treatment with glucocorticoids or polyamines.
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Metabolic zonation, that is, the spatial separation of the metabolic pathways among otherwise similar cells, potentially exists in two directions of the gut. The first direction, i.e. heterogeneity of the enterocytes along the proximal-distal axis of the organ, is pronounced during prenatal development, but is less obvious postnatally. The zonation in the second direction, i.e. along the crypt-villus axis, appears to be a characteristic of the citrulline/arginine biosynthetic pathway. In view of the fact that CPS, OTC, ASS, ASL and OAT should all be present within one enterocyte in order to sustain arginine biosynthesis, it may seem surprising, at first glance, that ASS and ASL mRNA and protein are absent from the enterocytes in the crypts and at the base of the villi. This finding implies that the "young" enterocytes, at the base of the villus, can only synthesize citrulline, whereas those near the top of the villus can not only synthesize citrulline, but can also convert it into arginine. Such a two-compartment system along the villus axis may enable the gut to simultaneously meet the needs for the synthesis of citrulline and arginine during the suckling period. This metabolic zonation is reminiscent of that in hepatocytes along the porto-central radius of the liver lobule (the smallest metabolic unit of that organ [52-54]).

We hypothesize that the capacity to produce arginine in the small intestine of sucklings is beneficial for several reasons. First, during this developmental period, the intestine has a very high growth rate and, thus, a high demand for amino acids. We already cited the study by Davis et al [12], implying a high net synthesis of arginine during the suckling period. Second, the surface area of the intestinal epithelium has a high potential not only for digestion and absorption of nutrients, but also for damage by noxious dietary substances and micro-organisms. To deal with the latter, the intestinal mucosa contains both physical barriers and immunological defences. During development, the need for a mucosal means of defence arises abruptly at birth. However, the specific mucosal immune system matures during suckling and becomes fully functional only after weaning [55]. Arginine has been identified as a potential immunomodulatory substance [56], partly because it is a substrate for nitric oxide synthase. Adult
enterocytes have been shown to express this enzyme [57,58], but a relation with a local intestinal arginine production in the neonate remains to be established.
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Chapter III

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

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Abbreviations used in this paper:

- CPS [EC 6.3.4.16] : carbamoylphosphate synthetase
- OTC [EC 2.1.3.3] : ornithine transcarbamoylase
- ASS [EC 6.3.4.5] : argininosuccinate synthetase
- ASL [EC 4.3.2.1] : argininosuccinate lyase
- A-I [EC 3.5.3.1] : hepatic arginase
- A-II [EC 3.5.3.1] : non-hepatic arginase
- OAT [EC 2.6.1.13] : ornithine aminotransferase
- SI [EC 3.2.1.48-10] : sucrase-isomaltase
- NOS : nitric oxide synthase
- FABPi : intestinal fatty acid-binding protein
- GALT : gut-associated lymphoid tissue
- ND : neonatal day, days after birth
- PP : Peyer’s patch
Summary

Background. Arginine is required for the detoxification of ammonia and the synthesis of proteins, nitric oxide, agmatine, creatine and polyamines, and 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 which 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 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 creatine. The expression of the very arginine-rich skin protein trichohyalin is not affected in transgenic mice. Finally, NOS-deficient mice did not show any of the features of arginine-deficiency.

Conclusions: 1) Enterocytes are important for maintaining arginine homeostasis. 2) Graded arginine deficiency causes graded impairment of skin, muscle and lymphoid development. 3) The effects of arginine deficiency are not mediated by impaired synthesis of protein or creatine.
Arginine is a precursor for the synthesis of proteins, NO, agmatine, creatine and polyamines, and an intermediate in the detoxification of ammonia. 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 amounts to approx. 20% of daily requirement (7), which barely meets daily demand under steady state conditions. A dietary source of arginine may therefore become necessary when demand increases under anabolic or catabolic conditions (7). For this reason, arginine is coined as a conditionally essential amino acid.

In rapidly growing suckling rats, the dietary supply of arginine via the milk does not suffice to 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 (5, 10-14) and do not express arginase (15). The intestine not only appears to play a crucial role in arginine biosynthesis in rapidly growing neonates like rodents and pigs, but also in neonatal humans, as destruction of the enterocytes in necrotizing enterocolitis also results in a selective decrease in circulating arginine (16). After weaning, argininosuccinate synthetase and argininosuccinate lyase, the enzymes that synthesize arginine from citrulline, disappear from the enterocytes, concurrent with the appearance of endogenous arginase (15), so that only the capacity to synthesize citrulline remains.

Arginine deficiency is known to cause a life-threatening hyperammonemia in preterm infants (17). Furthermore, arginine deficiency in growing animals is usually associated with growth retardation (12, 13, 15). However, it is not known if arginine
deficiency also affects developmental processes other than growth, and if so, what the quantitative relation between arginine deficiency and these sequellae is. Furthermore, the regulatory mechanisms underlying the development of these sequellae in growing mammals have to be solved to gain a better understanding of the role of arginine metabolism in normal growth, vessel-wall biology (18, 19) and immunological responsiveness (20, 21). We therefore developed a transgenic mouse model, which suffers from a selective decrease in circulating and tissue arginine as a result of overexpression of hepatic arginase I (A-I) in the enterocytes of the small intestine. The transgenic model highlights the importance of the small intestine for arginine metabolism in the suckling period. It further shows that a graded deficiency of arginine results in a graded deficiency in the growth and development of hair, muscle and immune system, but that these consequences do not arise from a deficient synthesis of protein, creatine and probably nitric oxide, or from an excess of ammonia.
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Animals

Mice. Mice were kept under environmentally controlled conditions (light on from 8:00 a.m. to 8:00 p.m.; water and rodent chow ad lib; 20-22°C, 55% humidity). Litters discovered in the morning were assigned ND0. Pups were weaned at 3 weeks of age. For generation of transgenics, mice of the FVB-strain were used. Spf-ash and NOS1-, 2- and 3-deficient mice were of C57/Bl6 background and purchased from Jackson Laboratories, Bar Harbor, Maine. Animal experiments were performed in accordance with the guidelines of the local Animal Research Committee.

Supplementation and weight curves. Nest-size was adjusted to 7 pups. Five mmol/kg of 150 mM arginine-HCl or creatine-monohydrate was injected subcutaneously on the back twice daily (9:00 a.m. and 4:00 p.m.). Control animals were injected with the same volume of 0.9% NaCl. Animals were weighed daily at 3:00 p.m. Each weight curve is based on repeated measurements of 10-16 animals. Male and female weight curves did not differ significantly.

Methods

Targeting arginase I expression to the enterocytes. The 4.3 kb construct used for generation of the transgenic animals (Fig. 1) is a chimera of the -1,178 to +28 bp fragment of the rat FABP1 promoter/enhancer element (22), 520 bp rat A-I cDNA, containing exons 1-5, and 2,400 bp of genomic rat A-I DNA, containing exons 5 to 8 of rat hepatic arginase (23). PCR of tail-tip DNA was carried out at 56°C using 5'-AAATGCCTACATGCTGTAGTCGG-3', complementary to nucleotide -218 to -196 of the FABP1 promoter/enhancer DNA as upstream primer, and 5'-CCAATTGCCATACTGTGGTCTCC-3', complementary to nucleotide +320 to +342 of the arginase I cDNA 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 PCR 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 (Fig. 2).
Fig. 1. Chimeric FABPI-arginase DNA construct used to generate F/A transgenic mice. Exons are indicated by black boxes. The 4.3 kb construct consists of a chimera of the -1178 to +28 bp EcoRI-BamHI rat intestinal FABPI promoter/enhancer fragment (61), the 520 bp PstI-BsrGI A-I cDNA fragment containing exons 1-5, and the 2400 bp BsrGI-HindIII fragment of genomic rat A-DNA, containing exons 6-8 (23).

**Determination of amino acid, creatine and ammonia concentration.** Pups were separated from their mother and kept at 37°C for 1 hour prior to sacrifice. After decapitation, blood was collected into heparin-containing tubes and centrifuged at 2,000 x g for 5 min at 4°C. Fifty μL of plasma was added to 4 mg of lyophilized sulphosalicylic-acid, centrifuged, and stored at -70°C. Tissue samples were collected, flushed in ice-cold PBS, rapidly frozen in liquid N₂ and stored at -70°C. Approximately 80 mg of tissue was added to 400 μL of a 5% sulphosalicylic acid solution, containing 300 mg glass beads with a diameter of 1 mm. The tissue was homogenized for 30 sec in a Mini Bead-Beater (BioSpec Products, Bartesville, OK) and centrifuged. Jejunum and plasma amino acids were determined by fully automated HPLC as described (24). 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 (25). Urea nitrogen was determined with diacetylemonoxime, as described (26).

Blood ammonia concentration was determined using the ammonia test kit (Menarini Diagnostics, Florence, Italy), according to the manufacturer’s instructions.
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Histology. For in situ hybridization, tissues were fixed in 4 % formaldehyde in PBS for 4 hours at 4\(^0\) C, and overnight at room temperature. In situ hybridizations were carried out as described (27). The A-I probe was the 768 bp PstI-StuI fragment of clone pARGr-2 (28). For immunohistochemistry, tissues were fixed in methanol/acetone/water (2:2:1; v/v) for 4 hours at 4\(^0\) C and overnight at room temperature, embedded in paraplast and sectioned at 7\(\mu\)m. After deparaffination, endogenous peroxidase activity was eliminated by treatment with 3 % H\(_2\)O\(_2\) in 50% methanol. Non-specific binding was blocked by incubation for 30 min in TENG-T (10mM Tris, 5 mM EDTA, 150 mM NaCl, 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), CPS (32) and SI (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-anti-peroxidase 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 hrs in methanol/DMSO (4:1) at 4 \(^0\)C with gentle agitation and incubated with a monoclonal FITC-conjugated antibody against murine VCAM-1 (1 \(\mu\)g/ml in PBS; PharMingen, San Diego, CA). After washing, tissues were incubated for 2 hrs at 37 \(^0\)C with HRP-conjugated anti-FITC antibodies (POD converter kit, Boehringer Mannheim) and stained with diaminobenzidine and H\(_2\)O\(_2\) in the presence of NiCl\(_2\).

Western blotting. Jejunal tissue and shaven skin was homogenized in 20 mM phenylmethylsulphony fluoride, 1 mM EDTA and 1 mM DTT. Twenty \(\mu\)g of protein (BCA assay, Pierce, Rockstone, IL) was separated on 10 % SDS polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). Membranes were blocked by overnight incubation in 10 % non-fat milk in PBS and subsequently incubated with primary antibodies. Goat anti-rabbit-IgG conjugated to alkaline phosphatase was used as secondary antibody.
**Quantification of serum immunoglobulin concentration.** Serum was collected from 8 and 18 day-old wild-type and homozygous F/A-2 littermates by decapitation. Total IgG concentration was quantified using an ELISA-based assay according to the manufacturer’s instructions (Southern Biotechnology Associates, Birmingham, AL).

**Arginase activity assay.** Intestinal tissue was homogenized in 40 volumes of 30 mM sucrose, 5 mM MnCl₂, 30 mM imidazole buffer (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. The assay mixture (200 µL) contained enzyme extract, 25 mM (guanidino-¹⁴C)-L-arginine (pH 9.5, 15 mCi.mol⁻¹; Dupont NEN, Boston, MA), and 75 mM glycine (pH 9.5). After 10 min at 37 °C, the reaction was stopped by loading the assay mixture on a cation-exchange column (BioRad, Richmond, CA). Urea was eluted with 1 ml of Tris-EDTA buffer, pH 8.5, and counted. Protein content was determined with the BCA assay.

**Statistics.** Weight data were fitted to a logistic growth curve (weight = weightₘₐₓ / (1 + e⁻ᵇᵗ⁻¹₀)). Fitting the curve in two stanzas (preweaning and postweaning) resulted in a significantly better fit (F-test on reduction of residual variation). To compare the resulting bi-phasic weight curves between genotypes and/or treatments a 99 % confidence interval was constructed both at 3 weeks and at 7 weeks. Biochemical data on arginase, amino acids, immunoglobulins and guanidino compounds were tested with a repeated measure analysis of variance (ANOVA) per 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 per 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 were tested with a Poisson distribution.
Results

*Intestinal arginase expression is associated with retarded body growth and retarded 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 (Fig. 2A-D). The animals are no longer distinguishable from wild-type animals by 6-7 weeks. Lines F/A -3 to -6 were indistinguishable from wild types. Histological analysis of homozygous F/A-2 skin at weaning revealed a severe distortion of hair follicles (Fig. 3A,D). Many hair bulbs lie too high in the subcutaneous tissue, contain keratohyalin grains and do not develop normally. Prominent keratin plugs clog the hair follicles. In general, the appearance is that of a delayed maturation of hair. However, the onset of sebaceous gland development appears 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, a potential target of arginine deficiency. However, immunohistochemical staining of skin sections (Fig. 3B and E) and Western-bLOTS of skin homogenates (Fig. 3C and F) did not reveal any differences in trichohyalin content, though we did observe absence of trichohyalin from the epidermis.

Mice show a biphasic growth curve in the first 8 weeks of life, with most rapid growth occurring immediately after birth and after weaning (Fig. 2E). The growth rate of homozygous F/A-1 and hemizygous F/A-2 mice is normal, but homozygous F/A-2 animals exhibit a pronounced retardation in body growth before weaning, which becomes discernible at 5 days after birth and which amounts to a 2-fold reduction in body weight at weaning. Since muscle is an important component of body weight, we analyzed the gastrocnemius muscle of 3-weeks-old homozygous F/A-2 mice histologically (Fig. 3G-J). The diameter of the myofibers is approx. 3-fold smaller than that of wild types, with many nuclei located in the periphery of the fibers.
Fig. 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 WT (wt/wt) mice in panel B, all at ND20. Note the difference in fur development and body size, in particular the similarity of F/A-1* and F/A-2* mice, and of F/A-1* and wild-type mice. Panel C: injection of arginine (right mouse), but not PBS (left mouse) restores fur development in F/A-1* mice. Panel D: injection of arginine causes recovery of F/A-2* mice (left animal), but only partial recovery of F/A-2* mice (right animal). Panel E: weight gain of F/A transgenics: WT ( ), F/A-1* ( ), F/A-2* ( ), F/A-2* ( ).
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2'''' mice have a significantly (p<0.01) lower body weight at weaning, but fully recover as adults. Panel F: weights of PBS- (▲) and arginine-injected (□) F/A-2'''' mice, compared to wild-type animals (◇). At ND21, arginine-injected F/A-2'''' mice showed significantly increased body weight, compared to PBS-injected F/A-2'''', but did not reach wild-type weight (for both: p<0.01). Panel G: Serum total IgG concentration in wild-type and F/A-2'''' mice at ND8 and ND18. Black bars, wild type; gray bars, F/A-2''''. Serum total immunoglobulin concentration is similar in both genotypes. For each measurement, serum of three mice was pooled. Values are ± SEM and based on three independent analyses. Panel H: weight curve of OTC-deficient spf-ash mice (▲) and wild-type C57/B16 mice (◇). At ND21, body weight of spf-ash mutant mice is significantly (p<0.01) lower than that of wild-type mice. Error bars depict a 99 % confidence interval of fitted growth curves.

Since no dystrophic features were observed, the picture is suggestive for active myogenesis.

Suckling homozygous F/A-2 mice appear to have a normal feeding behaviour and intestinal function. The absence of malnutrition was deduced from the presence of normally filled stomachs and a normal serum concentration of immunoglobulins (Fig. 2G), which are of maternal origin and have to be transported across the intestinal epithelium (37). Furthermore, the transgenic mice are not feeble but, instead, hyperactive (see Chapter IV).

The fur of the F/A transgenics resembles that of the OTC-deficient sparse-fur-abnormal-skin-and-hair (spf-ash) mutant mice (38). The growth rate of suckling spf-ash mice is also retarded significantly, but to a lesser extent than in F/A-2 mice (Fig. 2H).

Intestinal arginase expression selectively reduces arginine concentration

We confirmed that CPS and OTC are expressed in all enterocytes of suckling mice, and that ASS and ASL are only expressed in enterocytes on the distal half of the villi, as we have previously reported for the suckling rat intestine (15). As in suckling rats, endogenous arginase activity in the intestine does not become detectable until the third postnatal week in mice (Fig. 4A). Arginase activity in homozygous F/A-1 mice already amounts to 0.2 U/mg protein shortly after birth and rises to 0.7-0.8 U/mg protein in the second postnatal week.
Fig. 3. Skin and muscle development in wild-type and F/A-2^{+/-} mice. Panels A and D show haematoxylin and azophloxin-stained sections, and panels B and E sections of dorsal skin stained for the presence of trichohyalin in wild-type (A, B) and F/A-2^{+/-} (D, 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. Panels C and F: Western blot of skin extract stained for the presence of trichohyalin. Note similar concentration in both extracts. Panels G-J show haematoxylin and azophloxin-stained sections of gastrocnemius muscle of wild-type (G, H; transverse sections) and F/A-2^{+/-} mice (I, J; longitudinal sections) at ND20. 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.

Arginase activity in line F/A-2 follows a similar pattern, but at a 2-3-fold higher concentration. The accelerated rise in enzyme activity in F/A-1 and -2 is at least partly due to the rise in endogenous arginase. Apparently, the expression of A-I does not interfere with the expression of endogenous arginase. At day 20, endogenous arginase activity is highest in duodenum, whereas FABPi-driven A-I activity is highest in the jejunum and decreases towards the stomach proximally and the colon distally (Fig. 3).
4B). Hemizygous mice express arginase at an intermediate level between wild-type and homozygous mice (Table 1).

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**Fig. 4. Appearance and distribution of arginase activity in the small intestine.** Panel A: arginase activity in jejunal homogenates of wild-type and transgenic animals: F/A-2\(^{-/-}\) (△), F/A-1\(^{-/-}\) (■), wild-type (○). Panel B: distribution of arginase activity along the proximal-distal axis of the intestine in wild-type (white bars), F/A-1\(^{-/-}\) (gray bars), and F/A-2\(^{-/-}\) (black bars) mice at ND20. Note that the peak of FABP-driven A-I activity is found in the jejunum, whereas endogenous arginase expression is highest in the duodenum. Panels C-F show distribution of A-I mRNA and panels G-J of A-I protein in jejunal enterocytes of wild-type animals (C, G), F/A-1\(^{-/-}\) (D, H) and F/A-2\(^{-/-}\) (E, I), and F/A-3\(^{-/-}\) (F, J) at ND8. Note that lines F/A-1 and -2 express A-I in all enterocytes, whereas F/A-3 expresses A-I in approximately 5% of the enterocytes. Bar: 75 μm.

**Table 1. Intestinal arginase activity in F/A transgenic mice.**

Arginase activity (nmol/min/mg prot) was determined in hemizygous animals of the 6 available lines at ND12. Data represent the mean ± SEM of 6 mice. *: indicates statistical difference from wild type at p < 0.05.

<table>
<thead>
<tr>
<th>line</th>
<th>A-I activity (mU/g prot.)</th>
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<tbody>
<tr>
<td>WT</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>F/A-1</td>
<td>208 ± 3</td>
</tr>
<tr>
<td>F/A-2</td>
<td>450 ± 6</td>
</tr>
<tr>
<td>F/A-3</td>
<td>289 ± 31</td>
</tr>
<tr>
<td>F/A-4</td>
<td>169 ± 26</td>
</tr>
<tr>
<td>F/A-5</td>
<td>84 ± 17</td>
</tr>
<tr>
<td>F/A-6</td>
<td>221 ± 47</td>
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</tbody>
</table>
arginine in growth and development

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

In suckling wild-type mice, the concentration of arginine is 200-250 μM in plasma and 350-400 μmol/kg wet weight in jejunal tissues (Fig. 5A). In the homozygous suckling F/A-1 and -2 mice, plasma and jejunal arginine concentrations are reduced to, respectively, 90-110 and approximately 75 μM, and 110-140 and approximately 80 μmol/kg. Arginine concentration in F/A-1 mice returns to wild-type shortly after weaning. In homozygous F/A-2 mice arginine concentration also rises significantly after weaning in females (P < 0.01), but it remains depressed in males. Fig. 5B shows the relation between intestinal arginase activity and the corresponding arginine concentration in plasma or jejenum. A “sparse-fur” phenotype is only seen if intestinal arginase activity exceeds 0.6 U/mg protein, or if the circulating arginine concentration drops below 120 μM. Furthermore, small additional decreases in arginine concentration apparently suffice to cause a pronounced aggravation of the phenotype.

Arginine is the only amino acid of which the decrease in jejunum and plasma concentration corresponds with increasing transgenic arginase concentration in the enterocytes (Fig. 5B). Overexpression of arginase in the enterocytes is accompanied by a selective increase in the arginase product ornithine in jejunum of almost two-fold (p<0.01). Except a 45% and 20% increase in plasma glycine and tryptophan respectively, the plasma concentration of all other amino acids is similar in F/A and wild-type mice prior to weaning (not shown). The accumulation of ornithine is not associated with an increased tissue concentration of the polyamines spermine, spermidine or putrescine (not shown).
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Fig. 5. Arginine concentration in plasma and jejunum of wild-type and F/A-transgenic mice. Panel A shows arginine concentration in plasma and jejunum of wild-type (□), F/A-1+/− (□), F/A-1+ (□), F/A-2+/− (□) and F/A-2+ (□) mice as a function of age. Note that arginine concentration in transgenic animals is low during the suckling period, but recovers after weaning, except in male F/A-2+ mice. Panel B: plasma and jejunal arginine concentration as a function of jejunal arginase activity in wild-type, F/A-1+/−, F/A-1+ and F/A-2+ mice. On the Y-axis, plasma and jejunal arginine concentration are given in μM and μmol/kg wet weight, respectively.

The plasma concentrations of urea, the other arginase product, and of ammonia are not elevated (Table 2). Table 2 also shows that the concentrations of two other products of arginine metabolism, creatine and creatinine, known to be sensitive to decreases in plasma arginine (39, 40), are almost halved in both F/A-1 and -2 mice.
arginine in growth and development

The decreased creatine biosynthesis may well explain the increased concentration of its precursor glycine.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>F/A-1&lt;sup&gt;++&lt;/sup&gt;</th>
<th>F/A-2&lt;sup&gt;++&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Arginine</td>
<td>295±27</td>
<td>59±5*</td>
<td>63±8*</td>
</tr>
<tr>
<td>Ammonia</td>
<td>39±11</td>
<td>40±11</td>
<td>44±14</td>
</tr>
<tr>
<td>Urea</td>
<td>8.1±0.1</td>
<td>8±1</td>
<td>10±2</td>
</tr>
<tr>
<td>CT</td>
<td>406±33</td>
<td>218±2*</td>
<td>226±70*</td>
</tr>
<tr>
<td>CTN</td>
<td>18±2</td>
<td>11±1*</td>
<td>9±1*</td>
</tr>
</tbody>
</table>

Table 2. Products of arginine metabolism in F/A transgenic mice. Concentrations were measured in serum of 10 days old animals (n=3 pools of 3 animals each for each genotype). *: indicates statistical difference from wild type at p < 0.05

**Effect of arginine supplementation**

To demonstrate 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 neonatal day 3 onwards. Further 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 (Figs. 2C,D,F). Circulating plasma arginine concentrations in ND10 mice, 6 hours after the last injection, were 560, 120 and 90 μM 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. As creatine concentration is reduced twofold, we also tested supplementation with creatine, but this treatment was without effect.

**Maturation of intestinal mucosa in transgenics**

The normal absorption of maternal immunoglobulins (Fig. 2G) 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 (15). A-II and SI follow their normal developmental course in homozygous F/A-2 (Fig. 6A) and F/A-1 mice (not shown). However, ASS expression, which has become undetectable in wild types at ND20, is still detectable at this age in F/A-2 mice, but not thereafter (Fig. 6A). This temporary
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Persistence of ASS expression may well reflect a response to the limited bioavailability of arginine, since a similar feedback mechanism was recently reported for rat kidney (41).

We also assessed the pattern of expression of SI and ASS along the villus-crypt axis (Fig. 6B,C). Periweaning maturation of the small intestine starts 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 remains tightly associated with the disappearance of ASS, but follows a slightly slower course in the F/A-2 mice compared to 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 (42). SI and ASS, therefore, form a very useful set of markers to follow enterocytes maturation. In aggregate, the findings indicate that the maturation of the enterocytes in the F/A-2 mice only distinguishes itself from that in wild-type mice 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 (21). This prompted us to investigate the development of the immune system. At 3 weeks, the weight of spleen and thymus of homozygous F/A-2 mice is less than one fourth of that of wild-type animals of the same age (Table 3). Compared to wild-type pups of the same body weight (ND8), splenic weight is still decreased to 40%, whereas thymic weight is not significantly different.

<table>
<thead>
<tr>
<th></th>
<th>WT (ND21)</th>
<th>%</th>
<th>F/A-2</th>
<th>%</th>
<th>WT (ND8)</th>
</tr>
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<tr>
<td>Bodyweight</td>
<td>13.5 ± 1.0</td>
<td>4.7 ± 0.3</td>
<td>4.8 ± 0.2</td>
<td></td>
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<tr>
<td>Splenic weight</td>
<td>67 ± 10°</td>
<td>15 ± 0.6</td>
<td>38 ± 1°</td>
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<tr>
<td>Thymic weight</td>
<td>79 ± 7°</td>
<td>17 ± 0.4</td>
<td>24 ± 1°</td>
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</table>

Table 3. Splenic and thymic weights in wild-type and homozygous F/A-2 mice. Splenic and thymic weights of F/A-2°° mice are significantly lower than that of wild-type mice at ND21 (a,c; p<0.01). Splenic, but not thymic weight of F/A-2°° mice is also significantly reduced when compared to a weight-matched
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wild-type animal (c; p<0.01). %: organ weight as a percentage of body weight. Values are means ± SEM (n=12 for both groups).

Inspection of the intestines revealed that the development of Peyer's patches (PPs), the first macroscopically visible, morphological hallmark of the GALT, is also affected by arginine depletion. In the third postnatal week, 4-7 PPs are always seen protruding from the serosa of the small intestine of wild-type, homozygous F/A-1 and hemizygous F/A-2 mice (Fig. 7A). However, in a total of 23 small intestines of 18-days old homozygous F/A-2 animals, only 8 rudimentary PPs were identified macroscopically (Fig. 7B), instead of the more than 92 expected (p < 0.001). These remaining PPs were much smaller in size compared to the ones observed in wild-type small intestines (Fig. 6A',B').

Fig 6. Enterocyte maturation in wild-type and F/A-2+/− mice. Panel A: Western blots of jejunal tissue of wild-type and F/A-2+/− mice at ND14, 18, 20 and 24, probed with polyclonal antibodies against ASS, SI, A-I, and A-II.
Fig. 6, panel B: Expression of ASS and SI along the crypt-villus axis of the jejunum and ileum of wild-type (wt) and F/A-2<sup>+/−</sup> mice (+/+ ) at ND14, 16, 18, and 20. All sections were counterstained with haematoxylin.
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Fig. 6, panel B, continued.
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Fig. 6 Panel C: 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 wild-type (○), and F/A-2 (●) mice. Note the identical onset, but slightly protracted progress of enterocyte maturation in homozygous F/A-2 mice.

In homozygous F/A-2 pups that were supplemented with arginine, a normal number of normally sized PPs was found (Fig. 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 or not PP organogenesis is disturbed, we performed whole mount immunostaining of the small intestine of 6-day old pups for the presence of VCAM-1, the first marker of PP development (43). 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 small intestines of homozygous F/A-2 mice is indistinguishable from wild-type animals, demonstrating the presence of intact PP anlagen (Fig. 7D,E). In homozygous F/A-2 mice, PPs become macroscopically identifiable at 6-7 weeks 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. In order to reveal a possible mechanism for the effect of arginine on PP development, we investigated mice carrying null mutations of the three isoforms of the NOS gene (44-46) or the spf-ash mutation in the OTC gene. At ND18, the normal number and size of PPs was found in spf-ash-, as well as in nNOS-, iNOS-, and eNOS-deficient mice. This observation shows that the hampered development of PPs is not due to a deficient citrulline or nitric oxide production by any of these enzymes. In addition, the normal number and size of PPs in spf-ash mutants suggests that the failing development of PPs in F/A-2 homozygotes is not due to running. In aggregate, 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 levels of one of its metabolic products.

Fig. 7. Peyer's patch development in wild-type and F/A-2 transgenic mice. Panels A and B: the small intestine of a wild-type (A) and a F/A-2<sup>−/−</sup> (B) at ND18, with arrows pointing at PPs. PPs were not macroscopically identifiable in F/A-2<sup>−/−</sup> mice. Panel C: arginine injections normalize the appearance of PPs in F/A-2<sup>−/−</sup> mice (arrows). Panels D and E: whole-mount immunohistochemical staining of the small intestine of a ND6 wild-type (D) and a F/A-2<sup>−/−</sup> (E) animal for the presence of VCAM-1-positive PP anlagen (arrows). Note the presence of Peyer's patch anlagen in both genotypes. Panels A' and B': Haematoxylin and azophloxsin-stained sections of a typical PP in a wild-type (A') and an F/A-2<sup>−/−</sup> (B') mouse. Bars: 0.6 mm (panels A-E); 3 mm (panels D, E), 75 μM (panels A', B').
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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 demonstrated by the normal arginine concentration and phenotypic appearance of lines F/A-3 and -6, expression of the transgene in all enterocytes is necessary to elicit the characteristic phenotype. As demonstrated 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 (15). However, the results of the arginine injections show that the A-I overexpressing enterocytes also function as an effective arginine drain. Since F/A-2 adult males have a lower circulating concentration of arginine than females, they are apparently more sensitive to this effect than females. At present, 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 presently 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 weeks, plasma and intestinal arginine concentration in homozygous *spf-ash* mice are approximately 120 μM (47, 48). Combining these data with our own, we conclude that the sparse-fur trait develops when plasma arginine in the suckling period drops below 120 μM. Hampered muscle and immune development is only seen in homozygous F/A-2 mice and, therefore, develops when plasma arginine concentration drops below 80 μM. The lack of a linear relation between intestinal arginase levels and plasma arginine concentration in the respective transgenic lines may be due to induction of arginine biosynthesis elsewhere, e.g. in the kidney (41).
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Given the high $K_M$ of A-I, being in the millimolar range, such an extra-intestinal 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 (49) by specific neonatal Fc-receptors on the intestinal epithelium (37), shows that milk intake and absorption in F/A-2 mice is normal. The finding that the plasma and tissue concentration of all amino acids except arginine and ornithine are normal, and that the impaired growth of fur, muscle and PP development responds to arginine injections, further underscores the hypothesis that the observed developmental disorders solely result from an arginine deficiency.

**Causes of the development of the F/A phenotype**

Arginine is a precursor for the synthesis of proteins, creatine, agmatine, NO and polyamines and an intermediate in the detoxification of ammonia. We therefore evaluated these pathways for a possible cause of the F/A phenotype. Since the concentration of NH$_3$ and polyamines in F/A-2 mice is normal, these factors can be excluded. General protein synthesis is probably also not affected in our transgenic mice, as the $K_M$ of arginyl-tRNA synthetase for arginine is approx. 20 μM (50), i.e. well below the observed tissue arginine concentration in the F/A-2 transgenics. However, incomplete charging of arginyl-tRNA may affect proteins with a very high arginine content, especially if the arginines are adjacent (51). An example 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 tripletts (52). Unexpectedly, no decrease in trichohyalin content was found in tissue sections and Western blots of F/A-2 skin. Since the steady state concentration of this extremely arginine-rich protein is apparently not affected, we conclude that arginine deficiency does not exert its effects on fur development and, hence, on neonatal growth at the translational level.
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, since it was shown that metabolic deficiency of creatine can be corrected by substitution (53). 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 at present 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 (54).

The decreased spleen weight and the tardy development of PP s indicate that the selective decrease in circulating and tissue arginine concentration retards the development of the immune system. In accordance with the relatively normal thymusto-body weight ratio, 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 immuno-nutrient in enteral and parenteral nutrition in a clinical setting was already claimed 20 years ago (55, 56). Because nontoxic concentrations of nitric oxide are thought to enhance Th2-dependent immune responses (57), PP development was investigated in nNOS-, iNOS-, or eNOS-deficient mice. 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 three NOS isoforms are not available to conclusively prove or disprove NO involvement.

In aggregate, our findings indicate that the F/A phenotype develops as a result of a deficiency of arginine itself, rather than with a deficiency of products of arginine metabolism. Clearly, the 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, as homozygous F/A-2 mice share the combination of normal organogenesis, but disturbed maturation of PPs, in conjunction with normal lymph
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node development, with TNFα- and TNFR-1-deficient mice (58, 59). 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, as development of PPs, follicle-associated epithelium and M-cells was also found to be impaired in mice that lacked B-cells (60).

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Chapter IV

Effect of arginine deficiency on circulating and tissue amino acids and guanidino compounds, and on behavioral development

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Chapter IV

Abstract

Arginine is an intermediate of the ornithine cycle and serves as a precursor for the synthesis of nitric oxide, creatine, agmatine, and proteins. It is considered to be a conditionally essential amino acid, because endogenous synthesis only barely meets daily requirements. In rapidly growing suckling rats, endogenous arginine biosynthesis is crucial to compensate for the insufficient supply of arginine via the milk. Evidence is accumulating that the intestine rather than the kidney plays a major role in arginine metabolism in this period. Accordingly, ectopic expression of hepatic arginase in murine enterocytes by genetic modification induces a selective arginine deficiency. The ensuing phenotype, the severity of which correlates with the level of transgene expression in the enterocytes, could be reversed with arginine supplementation. We now report that these transgenic mice continue to suffer from an arginine deficiency after the arginine biosynthetic enzymes disappear from the enterocytes. Post-weaning catch-up growth in arginine-deficient mice is characterized by increased levels of all amino acids except arginine. Furthermore, amino acid concentrations, including arginine, are lower in adult male than in adult female transgenes. Decreases in the concentration of plasma and tissue arginine lead to corresponding decreases in most metabolites of arginine. However, the accumulation of the toxic guanidino compounds guanidinosuccinic acid and methylguanidine corresponds with arginine deficiency, possibly reflecting a higher oxidative stress under hypoargininemic conditions. Hypoargininemia is also associated with disturbed neuromotor behaviour, although brain levels of toxic guanidino compounds and ammonia are normal.
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Abbreviations

CPS [EC 6.3.4.16] : carboxamoylphosphate synthetase
OTC [EC 2.1.3.3] : ornithine transcarbamoylase
ASS [EC 6.3.4.5] : argininosuccinate synthetase
ASL [EC 4.3.2.1] : argininosuccinate lyase
A-I [EC 3.5.3.1] : hepatic arginase
A-II [EC 3.5.3.1] : non-hepatic arginase
AGAT [EC 2.1.4.1] : L-arginine-glycine transamidinase
ND : neonatal day, days after birth
Harg : homoarginine
ArgA : argininc acid
GAA : guanidinoacetic acid
CT : creatine
CTN : creatinine
MG : methylguanidine
G : guanidine
GABA : γ-aminobutyric acid
γ-GBA : γ-guanidinobutyric acid
β-GPA : β-guanidinopropionic acid
δ-GVA : δ-guanidinovaleric acid
α-K-δ-GVA : α-keto-δ-guanidinovaleric acid
GSA : guanidinosuccinic acid
α-NAA : α-N-acetylgarginine
Introduction

Arginine is an intermediate of the ornithine cycle and serves as a precursor for the synthesis of nitric oxide (NO), creatine, agmatine, and proteins (Wu and Morris, 1998). Arginine is not considered to be an essential amino acid. In adults, a major source of arginine biosynthesis from citrulline is the renal proximal convoluted tubule (Featherston et al., 1973; Levillain et al., 1992). Citrulline, in turn, is synthesized in the small intestine from glutamine and proline (Windmueller and Spaeth, 1981). Although arginine is not considered to be an essential amino acid in adult humans, its endogenous biosynthetic capacity only amounts to approx. 20% of daily expenditure (Visck, 1986). Hence, its bioavailability may become insufficient under conditions of increased demand, such as growth (Visck, 1986) and tissue repair (Thornton et al., 1997), or as a result of decreased dietary supply (de Lorgeril, 1998). For this reason, arginine is regarded as a conditionally essential amino acid.

In rapidly growing suckling rats, endogenous arginine biosynthesis is crucial to compensate for the insufficient supply of arginine via the milk (Davis et al., 1993). Evidence is accumulating that the intestine rather than the kidney plays a major role in arginine biosynthesis in the suckling period. Thus, the enterocytes of the small intestine express the enzymes required for arginine production from glutamine and proline (Wu, 1997) and do not express arginase (De Jonge et al., 1998). Furthermore, we have recently shown that overexpression of hepatic arginase in enterocytes induces an arginine deficiency (De Jonge et al, submitted). The intestine appears to play a similar role in human neonates as well, as destruction of the enterocytes in necrotizing enterocolitis also results in a selective decrease in circulating arginine (Zamora et al., 1997). In mice and rats, argininosuccinate synthetase and argininosuccinate lyase, the enzymes that synthesize arginine from citrulline, disappear from the enterocytes in the postweaning period, concurrent with the appearance of endogenous arginase (De Jonge et al., 1998), so that the role of the gut becomes confined to citrulline biosynthesis.
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The reduction in circulating arginine concentration in transgenic mice that express different levels of arginase I in their enterocytes ("F/A" transgenes) depends on the expression level of transgenic arginase. The degree of arginine deficiency correlates, in turn, with the degree of retardation of hair- and muscle growth, and the development of the lymphoid tissue (De Jonge et al, submitted). Expression of arginase in all enterocytes is necessary to elicit arginine deficiency. Phenotypic abnormalities are reversed by daily injections of arginine, but not creatine. Because the phenotypic symptoms largely disappear after weaning, the question arose whether circulating levels of arginine in relation to the other important amino acids normalize in our transgenic mice when intestinal arginine biosynthesis ceases after weaning.

Figure 1: Metabolic routes of arginine. Enzymes that catalyze the indicated reactions are given in italics. Arginase accepts arginine as well as homoarginine as a substrate. Similarly, AGAT transamidinates the guanidino group of arginine to glycine, but also to the other substrates indicated. The α-amino group of arginine can be transamminated to α-K-δ-GVA, and acetylated to α-NAA. GSA and MG are most probably formed from argininosuccinate (AS) and CTN, respectively, via a reaction with a reactive oxygen species.
We also investigated the accumulation of guanidino compounds, the metabolites of arginine that retain the guanidinium group. A schematic representation of the relation of arginine with the ornithine cycle and its guanidino metabolites is given in Fig. 1. Homoarginine, lysine, and homocitrulline (not shown) are homologues of arginine, ornithine, and citrulline, respectively, and as such are alternative intermediates of the ornithine cycle. A major metabolic route of the guanidino group of arginine is transamidination to glycine to yield guanidinoacetic acid (GAA), and subsequently creatine (CT) and creatinine (CTN). In addition to the physiological substrate glycine (α-aminoacetic acid), its homologues β-alanine (β-aminopropionic acid), γ-aminobutyric acid, and δ-aminovaleric acid can function as substrates of the enzyme AGAT, yielding β-guanidino propionic acid (β-GPA), γ-guanidino butyric acid (γ-GBA), and δ-guanidinovaleric acid (δ-GVA), respectively (Fritsch et al., 1999; Marescau et al., 1992b; Wyss and Kaddurah-Daouk, 2000). Furthermore, the α-amino group of arginine can be transaminated and acetylated, yielding α-keto-δ-guanidinovaleric acid (α-K-δ-GVA) and α-N-acetylarginine (α-NAA), respectively. Hydrogenation of α-K-δ-GVA produces argininc acid (ArgA) (Robin and Marescau, 1985). Methylguanidine (MG) forms after the reaction of an oxygen radical with CTN (Nakamura et al., 1991). Similarly, guanidinosuccinic acid (GSA) may be the product of the reaction of argininosuccinic acid and the action of an oxygen radical species (Aoyagi et al., 1999). Some guanidino compounds, in particular α-NAA, GSA and MG, are toxic and play a role in the pathology of renal (Tomida et al., 2000) and liver (Marescau et al., 1995) failure, in particular the metabolic (Horowitz et al., 1970) and neurological (da Silva et al., 1999; De Deyn et al., 2000; D'Hooge et al., 1996) consequences of these diseases. The neurotoxicity of guanidino compounds appears to be more pronounced in the growing than in the adult animal (D'Hooge et al., 1992; D'Hooge et al., 1994). The concentration of guanidino compounds was therefore determined in plasma and several tissues of 10-day old homozygous arginine-deficient transgenic mice, as well as in wild-type
controls. At this age, intestinal biosynthetic capacity for arginine is maximal (De Jonge et al., 1998) and the hypoargininemic phenotype of the transgenic mice most pronounced (De Jonge et al, submitted). The relation between circulating arginine concentration and the formation of guanidino compounds was investigated by arginine supplementation. Because of the neurotoxic potential of some guanidino compounds, like GSA and MG, and the possible long-term consequences of alterations of GABA and glycine levels on neurotransmission, we also determined the consequences of arginine deficiency on neuromotor development.
Chapter IV

Methods

Animals. Mice were kept under environmentally controlled conditions (lights on at 8:00 a.m., off at 8:00 p.m.; water and rodent chow ad lib; 20-22 °C, 55% humidity). Animal experiments were done in accordance with the guidelines of the local Animal Research Committee. Litters discovered in the morning were assigned ND 0. The animals were weaned at three weeks of age. For arginine injections, nest size was adjusted to 7 pups. Pups received a subcutaneous injection of 5 mmol/kg of arginine-HCl (150 mM), twice daily (9:00 a.m. and 6:00 p.m.) from postnatal day 5 onward. Controls were injected with saline (0.9 % sodium chloride).

Generation of transgenics. Intestinal fatty-acid binding protein (FABPi)/arginase I transgenic mice (F/A) were generated in the FVB-strain. Generation of the transgenics is described in detail elsewhere (De Jonge et al, submitted). Briefly, arginase I (A-I) was specifically expressed in enterocytes by coupling the A-I structural gene to the FABPi promoter/enhancer element. Two lines, F/A-1 and F/A-2, which express A-I in all enterocytes, but which differ 2-fold in the level of A-I expression (F/A-2 = 2 * F/A-1), were analyzed.

Tissue and blood sampling. Pups were separated from their mother and kept at 37° C for one hour prior to sacrifice. After decapitation, blood was collected into heparin-containing tubes and centrifuged at 2,000 x g for 5 minutes at 4° C. Fifty μL of plasma was added to 4 mg of lyophilized sulphosalicylic-acid, mixed, frozen in liquid nitrogen and stored at -70° C. Tissue samples were collected, flushed in ice-cold phosphate-buffered saline (PBS), rapidly frozen in liquid nitrogen and stored at -70° C until analysis.

Determination of amino acid and guanidino compound concentrations. Jejunum and plasma amino acids were determined by fully automated HPLC as described
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(van Eijk et al., 1993). Norvaline was used as an internal standard. For analysis of guanidino compounds, plasma was deproteinized with an equal volume of 20% trichloroacetic acid, followed by centrifugation at 16,000 x g at 4 °C. The supernatant was diluted and injected into an LC5001 amino acid analyzer (Biotronic, Maital, Germany), adapted for guanidino compound determination. For guanidino compound determination in tissue, approximately 40 mg tissue was homogenized (Tissue Tearor®, model 985-370 type 2, Biospec Products, Bartesville, USA) in 350 μl of ice-cold water. Thirty μl was taken for urea determination. The tissue homogenizer was washed immediately with 350 μl 30% trichloroacetic acid. the wash fluid was added to the first pool and the total was vortexed. The acetic protein complexes were precipitated by centrifugation at 20800 x g at 4 °C. Diluted supernatant was injected. Guanidino compounds were separated on a cation-exchange column using sodium citrate buffers and detected with the ninhydrin fluorescence method (Marescau et al., 1986). Urea nitrogen was determined with diacetylmonoxime (Ceriotti, 1971). Blood ammonia concentration was determined using the using the ammonia test kit (Menarini Diagnostics, Florence, Italy), according to the manufacturer’s instructions.

Behavioral tests. Mice of 3 months of age were used. Each animal was put in a separate cage (16x22 cm²) between 3 infrared photobeams connected to a microprocessor counter. Cage-activity was recorded between 16:00 p.m. and 8:00 a.m., and expressed as total number of beam crossings during the recording period. Open field activity was recorded in animals on a reversed dark-light schedule during the dark phase of the cycle, and their movements in a brightly lit area were tracked for 10 minutes using a computerized video tracking system (San Diego Instruments, USA). Exploratory activity was additionally assessed in a dark/light transition box consisting of a large illuminated (45x75 cm²) and a smaller (10x75 cm²), dark compartment. A dividing wall allowed transition between compartments through four evenly spaced 4-cm holes. Animals on reversed dark-light cycle were
placed in the box for 10 min (starting from the dark compartment) during the dark phase of the light cycle, and exploration of the illuminated area was registered using two photobeams (at 1 and 7 cm distance to the dividing wall) and a microprocessor-based counter.

For the wire suspension test, animals were put with their front paws on a taut steel wire (0.6 mm diameter), 46 cm above tabletop, and were to remain suspended for 120 s using their front paws only. Latency of the first slip and number of slips within the 120-sec test period were recorded. In the rotarod test, animals received a 1-min training trial at 4 rpm followed by four testing trials at 10-min intervals on an accelerating rotarod (Ugo Basile, Varese, Italy). Each testing trial consisted of a 5-min session during which the rod accelerated linearly from 4 to 40 rpm. The time the animals were able to stay on the rod was recorded automatically. Finally, gait characteristics were recorded using a runway apparatus. With their hind paws wetted with ink, the animals walked on a strip of paper down a brightly lit corridor (40 cm long, 4.5 cm wide) towards a dark goal box. Recordings were made in duplicate, and maximal distances between prints of left and right paws were measured from the tracks.

Passive avoidance learning was tested in a two-compartment step-through box. Animals on reversed dark-light cycle were put in the small (5x9 cm²) brightly lit compartment of the box. After 5 sec, the sliding door was opened, leading to the larger (20x30 cm²), dark compartment. Upon entrance into the dark compartment, the door was closed and animals received a slight electric foot shock (0.3 mA, 1 sec). Twenty-four hrs later, the procedure was repeated, and step-through latency was recorded up to 300 sec.

Statistical analyses. Biochemical data on amino acids and guanidino compounds were tested with a repeated measure analysis of variance (ANOVA) per compound group. Because of significant interactions between compound, genotype and age, a two-way ANOVA (amino acids; factors age and genotype) or a one-way ANOVA (guanidino compounds; factor genotype) was carried out per 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 if p<0.01.

For the amino acids with a significant age effect, a mean amino acid level was calculated by removing the multiplicative variation between amino acids (J.M. Ruijter et al, submitted). To this end, the correction factors for removal of variation were deduced from a matrix of between amino acid ratios. The mean amino acid level of adult wild type mice was set at 100. Thus, in the resulting mean amino acid all included amino acids carry equal weight.
Chapter IV

Results

Levels of circulating amino acids in wild-type and transgenic mice during postnatal development.

F/A-1 and F/A-2 are transgenic mouse lines that were genetically modified to express hepatic arginase (A-I) in their enterocytes in order to annul their capacity to synthesize arginine (De Jonge et al, submitted). The level of hepatic arginase in the enterocytes of transgenic line F/A-1 is 50% of that of line F/A-2.

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Table 1: statistical analysis (two-way ANOVA) of changes in amino acid levels in WT, F/A-1 and F/A-2 postnatal and adult mice. Significant effects (p<0.01) of genotype (G), age (A), or genotype-age interaction (I) are given in each column. * Indicates genotype effects which depend solely on age group 5-10 weeks.

To evaluate whether the apparent normalization of body and fur growth after weaning was associated with the normalization of circulating arginine levels, circulating amino acid concentrations, including arginine, were determined as a function of age. In suckling wild-type mice, the concentration of plasma arginine is 250 µM and gradually declines to 150-170 µM in adult male and female animals (Fig. 2A). In homozygous suckling and weanling F/A-1 mice, plasma arginine is reduced to 80-125 µM, whereas in homozygous F/A-2 suckling and weanling animals, plasma arginine averages only 70 µM.

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**Figure 2: Plasma amino acids as a function of age.**
The plasma concentration (μM) of arginine (panel A), ornithine (panel B), glycine (panel C), tryptophan (panel D), citrulline (panel E), and the mean of all other amino acids that decline with age (panel F) in wild-type, homozygous F/A-1 and F/A-2 mice in the preweaning period (1-2 weeks of age), in the periweaning period (3-4 weeks), in the adolescent period (5-10 weeks), and in the adult period (>10 weeks). Arginine is significantly decreased, whereas glycine, tryptophan, and ornithine plasma concentrations are increased in the F/A transgenics. Note the higher amino acid levels in the adolescent period of F/A-2 mice and the
higher arginine levels in adult female (open symbols) compared to male (closed symbols) F/A transgenic mice. Panel F represents the means of plasma concentrations of the amino acids that significantly decline with age (asparagine, glutamate, histidine, lysine, ornithine, serine, isoleucine, methionine, valine, threonine and tyrosine). Since the concentrations were corrected for multiplicative variation (see materials and methods), all amino acids weigh equally in this mean. No difference between sexes was found in suckling and adolescent mice. Asterisks indicate significant differences between male and female (P < 0.01).

No sex differences were found in suckling and weanling animals. In adult female F/A-1 and -2 mice, arginine concentration increases to 130-150 μM, whereas in adult transgenic males, it remains depressed (P < 0.01) at 110 and 80 μM in F/A-1 and F/A-2 mice, respectively (Fig. 2A).

In the preweaning period, arginine deficiency is associated with an increased concentration of glycine and ornithine (De Jonge et al, submitted). Table 1 shows the effects of genotype and age, as well as the interaction between genotype and age on circulating amino acid concentrations in wild-type, F/A-1 and F/A-2 mice from birth to adulthood. As shown in Fig. 2A, the decline in circulating arginine concentration depends strongly on genetic background (p<0.0005). In addition to ornithine (Fig. 2B) and glycine (Fig. 2C), circulating tryptophan (Fig. 2D), serine, threonine and phenylalanine concentrations are increased in transgenics. The significance of the genotype-related difference in ornithine, serine, threonine and phenylalanine (Table 1) depends solely on the increase in plasma amino-acid concentration in 5-10 weeks-old F/A-2 mice, as exclusion of this age group from the ANOVA abolishes significant differences between wild-type, F/A-1, and F/A-2 mice (Table I, genotype effects indicated by asterisks). Since the increased plasma concentration in 5-10 weeks-old F/A-2 mice is not limited to the amino acids mentioned (Fig. 2F and next paragraph), we felt justified to consider only tryptophan levels as being affected by the F/A genotype.

The Table further shows a significant age effect in the plasma levels of alanine, glutamate, methionine, ornithine, serine, taurine, threonine, histidine, lysine, tryptophan, asparagine, isoleucine, tyrosine, and valine, which is a decline with age in all mice (Fig. 2F), whereas the levels of citrulline (Fig. 2E), glutamine, and leucine do not change significantly in concentration during postnatal
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development (Table I). In wild-type mice, arginine, glycine and ornithine also exhibit an age-dependent decline, indicating that their circulating level is determined by both genotype and age. Fig. 2F shows the age-dependent decline of the mean concentration of the amino acids with a significant age effect. Two features are remarkable. First, the circulating concentration of these amino acids is significantly higher in fully-grown, adult transgenic females than in males, but not in wild-type control animals, showing that this gender difference is caused by the genotype, i.e. is the result of low circulating arginine concentrations earlier in life. Second, the circulating concentration of these amino acids is significantly higher in adolescent (5-10 weeks old) F/A-2 transgenic mice than in either wild-type or F/A-1 transgenic mice. The relatively high level of amino acids in adolescent F/A-2 mice coincides with the phase of catch-up growth from the growth retardation that they incur in the first 3 weeks of life (De Jonge et al, submitted).

Guanidino compounds under arginine deficiency

Arginine, homoarginine and urea (Fig.3A)

Arginine concentration of transgenic mice was significantly decreased in all organs analyzed. The effect was most pronounced in muscle, in which arginine levels decreased approx. 8-fold, and least in liver. No differences in urinary arginine concentration were observed. Despite the 2-fold difference in intestinal arginase expression and an associated aggravation of the phenotype (De Jonge et al, submitted), no striking differences in tissue and plasma arginine concentration between lines F/A-1 and F/A-2 were observed in this series of assays (cf. Fig. 2A). Arginine deficiency was accompanied by an even more pronounced decrease in the arginine homologue homoarginine. Unlike arginine, the decreased homoarginine levels resulted in a decline in urinary excretion. Furthermore, homoarginine levels in most organs were more affected in line F/A-2 than F/A-1. Significant increases in tissue urea concentration were only found in muscle and jejunum, that is, the
organ with the most pronounced drop in arginine concentration (muscle) and the organ with transgenic arginase activity (jejunum). Although urea levels tended to be higher in F/A-2 than F/A-1 and WT mice, plasma and urinary urea was not significantly increased in the transgenic animals.

![Figure 3A: Tissue, plasma and urinary concentration of guanidino compounds.](image)

Concentration of guanidino compounds in wild-type (black bars), F/A-1 (gray bars), and F/A-2 (white bars) mice at ND 10. Panel A: arginine (Arg), homoarginine (Harg) and urea. Tissue concentrations are given in μmol/kg, plasma and urinary concentrations in μM, except for urea concentrations, which are in mmol/kg or mM. <DL is below detection limit. Values are means ± SEM, with n = 6-21. Asterisks indicate significant difference (p<0.05) from wild-type means using a one-way ANOVA.
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Figure 3B: Tissue, plasma and urinary concentration of guanidino compounds.
panel B: the transamidination products, guanidinoacetic acid (GAA), β-guanidinopropionic acid (β-GPA), and α-guaninobutyric acid (γ-GBA).

Other products of arginine metabolism (Fig. 3B and C).
Arginine deficiency causes significant decreases of the transamidination products GAA, β-GPA, and γ-GBA in most tissues (Fig. 3B). However, plasma GAA level was not affected. Tissue and urinary concentrations exceed plasma concentrations, indicating active transport.
Figure 3C: Tissue, plasma and urinary concentration of guanidino compounds.

Panel C: the GAA products creatine (CT) and creatinine (CTN), the transamination product α-amino,δ-guanidinovaleric acid (α-K-δ-GVA) and its hydrogenation product argininic acid (ArgA).
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Figure 3D: Tissue, plasma and urinary concentration of guanidino compounds.

Panel D: guanidinosuccinic acid (GSA), methylguanidine (MG) and guanidine (G), and the transacetylation product α-N-acetylgarginine (α-NAA).
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The changes in transaminidation products are similar in F/A-1 and F/A-2 mice. Tissue creatine (CT) concentration in hypoargininemic animals follows the changes in GAA concentration, though in contrast to GAA, plasma and urinary CT levels are decreased in the transgenic animals (Fig. 3C). No effect of arginine deficiency was observed for the CT product CTN. The transamination product α-K-δ-GVA and its hydrogenation product ArgA are decreased in tissues and plasma, but not in urine (Fig. 3C). Generally, the decreases in α-K-δ-GVA, and argA were equal in F/A-2 and F/A-1 mice.

The formation of GSA, MG, G, and α-NAA (Fig. 3D)

GSA levels were increased in liver, kidney, jejunum and plasma of the transgenic mice. Furthermore, its excretion into urine was substantially increased. MG, which was only detectable in urine, was increased three-fold in F/A-2 mice. G, which was additionally detectable in jejunum and plasma, was increased only in the urine of the transgenic mice. Importantly, clear differences in the urinary excretion were observed between line F/A-1 and F/A-2, levels being the highest in the most affected line. In transgenic brain or muscle, GSA levels were unaltered, although brain arginine concentration is reduced to 40% and arginine in muscle to less than 15% of that of wild-type animals. In those tissues in which the α-amino acetylation product α-NAA was detectable, it was decreased in both transgenic lines, whereas its excretion into urine was increased.

Effect of arginine treatment

In order to demonstrate that the alterations in guanidino compounds in the transgenics are caused by arginine deficiency, we treated transgenic mice with twice daily subcutaneous injections of arginine, starting at ND5. Mice were sacrificed at ND10, 6 hours after the last arginine injection. At sacrifice, plasma arginine concentration was 560, 170 and 90 μM in wild-type, homozygous F/A-1 and F/A-2 mice, respectively, reflecting the influence of arginase activity in the
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transgenic intestine. The difference in plasma urea, GAA, CTN, α-K-δ-GVA, ArgA, GSA and G between F/A-2 animals on the one hand and the wild-type and F/A-1 animals on the other, shows that urea production mirrored circulating arginine concentration. In a few cases (CTN, α-K-δ-GVA and G), the plasma guanidino compound concentration in F/A-1 animals was also significantly lower than that in wild-type animals. Similar differences were found for β-GPA, γ-GBA and CT, but these numbers did not reach significance. Urinary CT excretion paralleled the corresponding circulating compounds. The urinary data also reveal that the bulk of the injected arginine is metabolized by arginase and excreted as urea in both control and transgenic mice. Whereas the urinary excretion of most compounds increased or remained constant upon arginine supplementation, that of GSA decreased in the transgenic mice. In summary, the observed response of plasma and urinary guanidino compound concentration to arginine supplementation indicates that the altered concentrations of guanidino compounds that are found in F/A mice, reflect the decreased availability of arginine. Because of the rapid establishment of the equilibriums (< 6 hours), plasma guanidino compound concentrations can be interpreted as fluxes.

Behavioral alterations in adult F/A transgenics

Cerebral arginine concentrations are decreased to 50% and 40% in F/A-1 and F/A-2 transgenics, respectively. These decreased levels of arginine may limit cerebral NO synthesis, as has been shown for endothelial cells (Arnal et al., 1995) and activated macrophages (Norris et al., 1995), and decreased NO synthesis has been reported to impair long-term potentiation (Son et al., 1996). Furthermore, in both transgenic lines the cerebral content of creatine is decreased to 70%, while the concentration of the purported neurotoxins GSA and ammonia (WT: 39 ± 11, F/A-1 40 ± 11, F/A-2 44 ± 14 µM), are not altered. In view of this potential modification of metabolism in the brain of F/A transgenic mice, it was decided to evaluate its possible consequences for brain function.
Table 2. Behavioral assessment in wild-type and transgenic groups.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>F/A-1</th>
<th>F/A-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cage activity</strong></td>
<td>beam crossings</td>
<td>8,400 ± 700</td>
<td>10,000 ± 500*</td>
</tr>
<tr>
<td><strong>Open field activity</strong></td>
<td>path length (cm)</td>
<td>3,900 ± 300</td>
<td>4,400 ± 400</td>
</tr>
<tr>
<td></td>
<td>corner entries (cm)</td>
<td>72 ± 4.0</td>
<td>86 ± 6.0</td>
</tr>
<tr>
<td><strong>Wire suspension</strong></td>
<td>latency first slip (s)</td>
<td>104 ± 11</td>
<td>55 ± 14*</td>
</tr>
<tr>
<td></td>
<td>slips/120 s</td>
<td>0.2 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td><strong>Rotarod (trial 4)</strong></td>
<td>time on the rod (s)</td>
<td>271 ± 14</td>
<td>232 ± 19</td>
</tr>
<tr>
<td><strong>Gait test</strong></td>
<td>maximal span (cm)</td>
<td>6.4 ± 0.2</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td><strong>Passive avoidance learning</strong></td>
<td>step-through latency (s)</td>
<td>300 ± 0.0</td>
<td>238 ± 32*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, n=10-11. Asterisks indicate significant difference from wild-type using Dunnett's multiple comparison test after significant between-genotype effect (ANOVA).

Table 2 shows that both F/A transgenic lines were impaired in several behavioral tests, and that the F/A-2 group generally deviated more from wild-type than the F/A-1 group. Cage and open field activity were increased in the F/A transgenic mice compared to controls. In the open field tests, ambulatory activity measures like path length and corner entries, differed significantly between the groups, whereas exploratory activity measures, like entries and dwell in center did not. Accordingly, transitions between the compartments of the dark-light box were not significantly different between the groups. Together, these tests demonstrated general hyperactivity in the transgenic mice, with the F/A-2 line being most severely affected. This hyperactivity could not be reduced to a qualitative activity difference (e.g., increased exploration). Several additional, more specific neuromotor abilities were tested. Wire suspension (grip strength and endurance) and rotarod performance (equilibrium and coordination) showed significant decreases in F/A transgenics. Analysis of their gaits revealed that F/A mice walked with shorter paces than controls. Finally, passive avoidance learning was impaired since the
step-through latency was decreased in F/A-1 and F/A-2 transgenic mice compared to controls.
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Discussion

Arginine deficiency and amino acids

Endogenous arginine biosynthesis is a typical example of interorgan metabolic cooperation. The intestine is special in that it has the unique capacity to synthesize citrulline, whereas many other tissues express argininosuccinate synthetase and argininosuccinate lyase to metabolize the citrulline originating from the intestine or from local NO synthesis to arginine. In the adult mammal, 60% of arginine formation from citrulline occurs in the kidney (Yu et al., 1996). However, the intestine rather than the kidney appears to play a major role in arginine metabolism in the suckling period (Herzfeld and Raper, 1976; Hurwitz and Kretchmer, 1986). Accordingly, the F/A transgenic mice, which express different levels of arginase in their enterocytes, suffer from a graded reduction in circulating arginine concentration, in particular during the suckling period. The functional significance of the intestine in arginine metabolism is not yet fully understood, but the degree to which hair and muscle growth (De Jonge et al., submitted), and B-cell maturation (De Jonge et al., submitted) are disturbed in F/A transgenic mice, is directly related to the level of arginase that accumulates in the enterocytes. We now report that circulating arginine levels in F/A transgenes remain low after the capacity to synthesize arginine in the gut ceases to exist, probably due to the continued breakdown of circulating arginine by intestinal arginase. The accelerated disappearance of injected arginine in the transgenic mice shows that the capacity to catabolize circulating arginine already exists in suckling F/A mice. For this reason, we do not know at present to what extent overexpression of arginase in the enterocytes depresses circulating arginine by interfering with local biosynthesis or by increasing catabolism of circulating arginine. We also do not know why circulating arginine levels are lower in male than in female transgenic mice.

Arginine belongs to the group of amino acids, of which the circulating concentration declines concurrent with the declining growth rate in the course of postnatal development. The expression of arginase in enterocytes is sufficient to completely abolish the high circulating arginine levels in the suckling period,
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suggesting that the observed phenotype was a direct consequence of arginine deficiency. The mechanism by which low arginine concentrations prevent hair and muscle growth, remains to be solved. Because extremely arginine-rich proteins such as trichohyalin, are present in normal amounts in F/A-2 mice (De Jonge et al, submitted), the most obvious explanation, that is the precursor function of arginine for protein synthesis cannot account for the phenotype of hypoargininemia. Arginine is also known as a secretagogue of growth-promoting hormones, such as growth hormone (Barbul et al., 1983) and insulin (Palmer et al., 1975), via mechanisms dependent, as well as independent of the production of nitric oxide from arginine (Jun and Wennmalm, 1994). However, animals of the F/A-2 line show catch-up growth after weaning, even though the concentration of circulating arginine remains low. The association of this delayed growth spurt with the 50% increased concentration of virtually all amino acids, is probably not a coincidence. Together, these data suggest that the ratio between arginine and the other members of the age-dependent group of amino acids determines whether or not growth is inhibited. This hypothesis is supported by the difference in growth behavior of F/A-1, which grow normally, and F/A-2 mice, which are severely affected, even though the additional decrease of arginine levels is modest. We are presently following these and other leads.

Another intriguing observation is the rise in circulating arginine levels in adult F/A females, but not in males. The effect is more pronounced in the F/A-2 than in the F/A-1 line. Since the intestinal arginase expression is similar in both sexes and since arginine biosynthesis by e.g. the kidneys can be adaptively increased (Prins et al., 1999), this finding suggests that females are able to mount a more effective adaptive increase in arginine biosynthesis than males. Alternatively, the requirements for arginine may be higher in males than in females, e.g. for creatine synthesis, because creatine accumulates to very high levels in testis and muscle ((Moore, 2000); see also next paragraph). Since this gender difference is also observed for the other age-dependent amino acids in the
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F/A transgenics, the cause may also be more general, e.g. a lower rate of amino acid catabolism in female arginine-deficient animals. Irrespective, this gender difference may well be of clinical importance, since a common disease such as atherosclerosis, has been hypothesized to represent an arginine-deficiency disease (Cooke, 1998) and since males are more prone to develop atherosclerosis than females (Clarkson et al., 1985). In view of the atheroprotective effect of estrogens (Elhage et al., 2000), future studies should also address the interaction between estrogens and arginine metabolism.

The instant hydrolysis of newly synthesized and circulating arginine by transgenic arginase in the enterocytes of suckling F/A mice leads to a drop of circulating and tissue arginine levels. As described in Chapter III, arginine deficiency was associated with an increase in the levels of ornithine and glycine, when only the preweaning period is considered. The inclusion of the postweaning and adult mice in the current analyses reveals genotype-dependent increases in the levels of ornithine, glycine, serine, tryptophan, threonine and phenylalanine. The 40% increase in circulating glycine may well be due to the diminished synthesis of creatine. Creatine plays an essential role in the energy metabolism of muscle, nerve and testis (Walker, 1979), and accounts for a sizable portion of arginine catabolism (Visek, 1986). Moreover, GAA levels in brain, liver and kidney reach maximum levels in the suckling period (Watanabe et al., 1985), underscoring the importance of arginine metabolism for CT synthesis in this period. The diminished synthesis of creatine is due to the decreased concentration of available arginine and not to feedback inhibition of the transaminidase reaction by creatine or ornithine. Conceivably, ornithine concentration could be increased as a result of the increased flux through arginase, but increased ornithine levels were only observed in intestine, where creatine levels are not altered and transaminidase activity is virtually absent (McGuire et al., 1986). The genotype-related increases in serine, threonine and phenylalanine were found to be solely due to their increased level at 5-10 weeks concentrations in F/A-2 animals. Since all amino acids except arginine were elevated at this time (Fig 2F), we do not attach too
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much importance to it. At present, we have no explanation for the increase in circulating tryptophan. Interestingly though, a similar increase in tryptophan was found in brain of OTC deficient spf mice (Bachmann and Colombo, 1984).

The absence of an increased urea concentration in tissue, plasma or urine is at first glance surprising in view of the overexpression of arginase in the enterocytes and the ensuing consumption of body arginine content. However, as also shown by the extremely low urinary urea content, amino acid catabolism in the suckling period is very low (Blommaart et al., 1993). Hence, dietary and endogenously synthesized arginine largely ends up in protein or creatine. Loss via the creatine/creatinine pathway virtually stops in the F/A mice. Arginine content in rodent protein is 3.5-4% (Davis et al., 1993), so that arginine degradation in the intestine only represents a tiny fraction of the total substrate for urea synthesis.

Arginine deficiency and guanidino compounds

The decreased arginine availability in F/A mice decreased the concentration of guanidino compounds that form via the transaminidation pathway, but caused an accumulation of GSA, MG and G. Strictly speaking, our data do not allow conclusions about fluxes. However, we observed that 6 hours after an arginine injection, the concentration of arginine and the arginine metabolites urea, GAA, β-GPA, γ-GBA, CTN, α-K-δ-GVA, ArgA, GSA, and G were still substantially increased in plasma of wild-type mice, but were similar to the corresponding control animals in F/A-2 mice, with animals from the F/A-1 line taking an intermediate position. The plasma levels of these compounds therefore correspond with the actual availability of arginine instead of with the administered arginine load, in other words, are a parameter for flux. The exception is homoarginine, which is not a metabolite of arginine. This conclusion implies that the decline in arginine concentration, and not the accumulation of transaminidation products (McGuire et al., 1984; Sipila, 1980), determines the decrease in flux through transamidinase.
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The increase in tissue, plasma and urinary GSA levels and urinary G levels corresponds with the increased severity of arginine deficiency. The higher concentration of GSA and G in the urine of transgenic mice upon arginine loading confirms that the formation of these compounds is enhanced under arginine-deficient conditions. Increased α-NAA, GSA, MG and G levels are also observed in renal failure (Cohen et al., 1968; De Deyn et al., 1986; Marescau et al., 1997), subtotal nephrectomy (Al Banchaabouchi et al., 1998; Levillain et al., 1995), endotoxin treatment (Deshmukh et al., 1997), and after consumption of an arginine-free diet by strict carnivores (Deshmukh and Rusk, 1989). As in the diseased states mentioned, the contribution of GSA and/or MG and G to the development of the arginine-deficient phenotype remains to be established.

It has been proposed that GSA is a transamidination product of arginine, when aspartate, instead of glycine, is the amidine donor (Cohen et al., 1968). However, since the flux through transaminidase is slowed down due to arginine deficiency in F/A mice, this explanation is not favored. GSA concentrations positively correlate with plasma urea (Marescau et al., 1992a). Urea, in turn, inhibits argininosuccinate lyase (Menyhart and Grof, 1977), suggesting that argininosuccinate may accumulate locally. The hypothesis that GSA, as well as MG, form upon interaction of argininosuccinate and creatine with free radicals (Aoyagi et al., 1996) is therefore more attractive, the more since it has been shown that free radicals form in the neonatal intestine (Musemeche et al., 1993), and probably elsewhere under catabolic conditions.

Arginine deficiency and behavioral deficits

F/A transgenic mice displayed hyperactivity as well as several more specific deficits in coordinated neuromotor abilities. Since passive avoidance learning was also impaired in these animals, although the motor requirements of this task are minimal, these deficits are probably not caused by muscular problems. Behavioral deficits, comparable with the ones seen in the F/A mice, have been associated with increased levels of urea and CTN in nephrectomized mice (Al Banchaabouchi et
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al., 1999). Uremic toxins, in particular GSA, affect cerebral neurotransmitter systems and are thought to cause the psychomotor deficits seen in uremic encephalopathy (D’Hooge et al., 1996). Behavioral alterations, similar to those seen in F/A mice, were found in spf-mutant mice (Batshaw et al., 1995). Unlike F/A mice, these mice suffer from increased ammonia levels. However, brain ammonia, urea, CTN and GSA concentrations are unchanged in F/A mice, virtually excluding these factors as causes of their neuromotor deficits. Similarly, GSA levels were not elevated in brain of spf mice (Batshaw et al., 1995). However, we cannot presently exclude that the slightly elevated circulating levels of tryptophan, a precursor for cerebral production of serotonin and quinolinate, a known excitotoxin (Batshaw et al., 1993), are responsible for the observed neuromotor deficits in F/A mice. Likewise, elevated brain tryptophan levels in spf mice (Bachmann and Colombo, 1984) have been associated with a two-fold increase in brain quinolinic acid, neuropathology (Robinson et al., 1995), and impaired passive avoidance (Batshaw et al., 1995).

As arginine is required for NO synthesis in neurons, the low level of circulating arginine in the F/A mice may be limiting for NO synthesis. Limitation of cerebral NO synthesis has been shown in spf mice, which also suffer from arginine deficiency (Ratnakumari et al., 1996). A hampered NO synthesis impairs synaptic plasticity, motor coordination and memory functions (O'Dell et al., 1994; Son et al., 1996). Neuronal NOS-deficient mice show behavioral alterations, but unlike F/A transgenics, wire suspension, open field activity, pole equilibrium and several other neuromotor tests were not altered in these animals (Nelson et al., 1995). Furthermore, mice lacking nNOS, especially males, were reported to be extremely aggressive, but we did not observe any signs of this aggressive behavior in F/A mice.
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Conclusion

F/A transgenic mice suffer from a life-long deficiency in circulating arginine and lasting behavioral deficits. Despite a low circulating arginine level, F/A-2 transgenics show a catch-up growth after weaning, which is temporally related to increased circulating levels of all other amino acids. Arginine levels in adult F/A females are higher than in adult F/A males, possible as a result of the extra requirement for CT synthesis in muscle and testis. Low levels of arginine lead to a decrease in the flux through transaminidase and accumulation of GSA and G, probably as a result of a higher oxidative stress under hypoargininemic conditions.

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Arginine deficiency affects early B cell maturation and lymphoid organ development in transgenic mice.

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**Abbreviations**

A-I, hepatic arginase
A-II, non-hepatic arginase
IEL, intraepithelial lymphocyte
IFNγ, interferon γ
IL, interleukin
LPL, lamina propria lymphocyte
MLN, mesenteric lymph node
NOS, nitric oxide synthase
OTC, ornithine transcarbamoylase
PLN, peripheral lymph node
PP, Peyer’s patch
Spf-ash, sparse-fur and abnormal skin and hair
TNFα, Tumor Necrosis Factor α.
Arginine deficiency impairs B cell maturation

Abstract
Arginine is required for the synthesis of protein, NO, creatine, agmatine and polyamines and the detoxification of ammonia. Another purported role of arginine is its immunosupportive function. In suckling mammals, arginine is synthesized in the small intestine. The functional relation between intestinal arginine metabolism and lymphocyte maturation and lymphoid organ development was investigated in transgenic mice that express hepatic arginase in their enterocytes. In the highest expressor line, F/A-2, arginine concentration in plasma and tissues is selectively decreased to 30-35 % of controls. B cell numbers were reduced in the peripheral lymphoid organs of these mice. The number and size of Peyer’s patches in the intestine was drastically reduced. Analysis of the bone marrow revealed that arginine deficiency impaired B cell maturation at the transition from the pro- to pre-B cell stage. In F/A-2 serum, immunoglobulin M level is decreased, but a normal B cell proliferative response can be induced. In contrast, the number and phenotype of T cells, found in the intestine, spleen or thymus of F/A-2 transgenic mice is normal. None of these phenotypes were found in NOS- or OTC-deficient Spf-ash mice. The findings demonstrate that a decrease in circulating arginine affects early B cell maturation.
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Introduction

Arginine is a precursor for the synthesis of protein, NO, creatine, agmatine and polyamines and an intermediate in the detoxification of ammonia via the ornithine cycle. Arginine is not considered to be an essential amino acid. In adults, it is produced in the kidney (1, 2) from circulating citrulline (3), which, in turn, is synthesized by enterocytes of the small intestine (4). In adult humans, the endogenous biosynthetic capacity for arginine amounts to approx. 20% of daily expenditure, i.e. is relatively small in comparison with its daily requirement (5). Hence, a dietary supply may become indispensable under conditions of increased demand, such as growth (5) and tissue repair (6), or as a result of decreased dietary supply (7). For this reason, arginine is coined as a conditionally essential amino acid.

An intriguing feature of the nutritional benefit of arginine is its immunosupportive effect, especially under catabolic conditions (8, 9). In studies on wound healing, arginine was already identified as an immunonutrient more than 20 years ago (10, 11). On this basis, it is added to postoperative supplemental formulas at doses as high as 100 g per kg formula (12). However, the molecular mechanism underlying the beneficial effect of arginine on lymphocyte biology has remained unclear.

In rapidly growing suckling rats, endogenous arginine biosynthesis is crucial to compensate for the insufficient supply of arginine via the milk (13). In this period, the intestine rather than the kidney plays a major role in arginine biosynthesis (14-16). The selective decrease in circulating arginine in neonatal patients who suffer from necrotizing enterocolitis, suggests a similar role of the enterocytes in arginine metabolism in early human development as well (17). On this basis, we developed a transgenic mouse model that suffers from a selective deficiency in arginine (18). In these mice, arginase I (A-I, EC 3.5.3.1) is selectively overexpressed in the enterocytes of the small intestine by coupling the A-I gene to the intestinal Fatty-Acid Binding Protein promoter/enhancer (FABP) (19). Hence, these mice can no longer accumulate arginine in their enterocytes, so that arginine levels decline to 30% of control levels. In the current study, we employed this
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We demonstrate that arginine-deficiency affects early B cell maturation in the bone marrow, but does not affect thymic T-cell development. In secondary lymphoid organs, like spleen and Peyer's patches, the number of B cells is decreased, though resident B cells are capable to proliferate normally upon in vitro stimulation. In addition, the plasma level of IgM is reduced in transgenic animals. These phenotypic abnormalities were not observed in NOS-deficient mice or in Spf-ash mutant mice, which, due to a mutation in the ornithine cycle enzyme OTC (20), suffer from a decreased circulating arginine level resulting from the impaired synthesis of the arginine precursor citrulline. These findings suggest a direct involvement of arginine on B cell maturation.
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Animals

Transgenics. A chimeric construct of the intestinal Fatty Acid Binding Protein promoter/enhancer element and hepatic arginase minigene was used to generate transgenic mice in the FVB strain background (18). The 4.3 kb construct consisted of the -1178 to +28 bp EcoRI-BamHI fragment of the rat intestinal Fatty Acid Binding Protein (FABPi) promoter/enhancer element, the 520 bp PstI-BsrGI fragment of the rat A-I cDNA, containing exons 1 to 4 of rat hepatic arginase and the 2400 bp BsrGI-HinDIII fragment of genomic rat A-I DNA, containing exons 5 to 8 of rat hepatic arginase. The line with the highest expression level of arginase, designated F/A-2 (18), was used in the current investigation. Spf-ash and NOS1-, 2- and 3-deficient mice were of C57/B16 background and purchased from Jackson Laboratories, Bar Harbor, Maine. Litters discovered in the morning were assigned neonatal day 0. The animals were weaned at three weeks of age. Mice were kept under environmentally controlled conditions (lights on at 8:00 a.m., off at 8:00 p.m.; water and rodent chow ad lib; 20-22 °C, 55% humidity). Animal experiments were done in accordance with the guidelines of the local Animal Research Committee.

Tissue and blood sampling. Pups were separated from their mother and kept at 37 °C for one hour prior to sacrifice. After decapitation, whole-body blood was collected and centrifuged for 2 minutes at 4 °C. Serum was kept at -70 °C until analysis. Tissue samples were collected, flushed in ice-cold PBS and rapidly frozen in liquid nitrogen. Tissue samples were kept at -70 °C until analysis.

Methods

Cell preparation and culture. Intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were isolated as described (21). In short, the small intestine was isolated, trimmed free of fat and mesentery, and flushed with ice-cold PBS to remove fecal content. The small intestine was cut into 1 cm fragments, and incubated for 30 min at 37 °C in calcium-free medium in the presence of 1 mM EDTA and 1 mM DTT, followed by a 30 min incubation in RPMI culture medium, supplemented with 20 U/ml DNase, to remove enterocytes and IELs. For
Arginine deficiency impairs B cell maturation

subsequent isolation of LPLs, Peyer's patches were removed, and a cellular suspension was obtained from the remaining intestinal tissue using an automated mechanical tissue desegregation device (Medimachine System, Dako, Denmark). For preparing spleen, thymus and mesenteric lymph node cell suspensions, 40 μm filter cell strainers (Becton/Dickinson Labware, New Jersey, USA) were used. Bone-marrow cell suspensions were obtained by flushing femurs and tibias with Dulbecco's Modified Eagle medium (DME). Cells were suspended in DME containing 10 % Fetal Calf Serum (FCS) (Gibco/BRL, Paisley, Scotland) and counted. Spleen and intestinal cell suspensions were centrifuged in sterile Ficoll (Pharmacia, Uppsala, Sweden). Mononuclear cells were transferred to cold PBS, containing 0.5% bovine serum albumin, 0.3 mmol/l EDTA and 0.01% sodium azide (Sigma) for flow cytometry, or to DME supplemented with 10 % FCS for culture.

In vitro stimulation and proliferation. Triplicate cultures of splenic cells containing $10^5$ B cells were started with LPS (E.Coli EH100, 15 μg/ml, a gift from Dr. C. Galanos), goat F(ab') anti-mouse μ chain (20 μg/ml, Jackson ImmunoResearch), anti-CD40 antibody (clone HM40-3, 4 μg/ml, Pharmingen) or IL 4 (500 U/ml, Pharmingen), either alone or in combination for the indicated times. (³[H]-Methyl-) Thymidine was added 6 hours before harvesting the cells.

Flow cytometry. The following antibodies were used for detection of lymphocyte surface markers. Monoclonal antibodies to mouse CD3ε, CD4, CD8, CD19, CD21, CD23, CD24, CD25, CD43, integrin αIEL and IgD were either from our collection or purchased from Pharmingen or Southern Biotechnology Associates (Birmingham, Alabama). They were used unlabeled or biotin-labeled, followed by staining with appropriately labeled secondary antibodies or streptavidin, or they were used as such, but covalently labeled with an appropriate fluorochrome. Goat F(ab')2 anti-mouse IgM and goat F(ab')2 anti-rat IgG were purchased from Caltag Lab (Burlingame, CA). Fluorochromes used were FITC, phycoerythrin (PE), Cy-5, and a tandem dye of Cy-5 and PE. Non-specific binding of goat anti-rat antibodies was blocked by incubation with 5 % normal mouse serum. Cells were analyzed by
flow cytometry using a FACSScan® or FACSCalibur flow cytometer, in conjunction
with the CellQuest® software (Becton Dickinson, Mountain View, USA).

Histology and immunohistochemistry. For histological analyses of spleen and PPs,
tissues were quick-frozen in Tissue-Tek OCT compound and sectioned at 6 μm.
Sections were fixed in dehydrated acetone for 10 minutes at room temperature, air
dried and incubated with rat anti-mouse antibodies. Polyclonal rabbit anti-mouse
IgM was purchased from DAKO, Tilburg, The Netherlands. The following rat anti-
mouse mAbs were used: anti-CD3ε (clone KT-3), anti-CD45R (B220), anti-
MAdCAM-1 (clone MECA-367) and anti-SER-4 (marginal zone macrophages).
After washing in PBS, sections were incubated with the appropriate dilution of the
peroxidase-conjugated second step reagent (Jackson ImmunoResearch, San
Francisco, CA) in 5 % newborn calf serum and 5 % normal mouse serum in PBS.
Double labelings were performed as described (22). In short, acetone-fixed cryostat
sections (6 μm thick) were incubated with a rat anti-mouse MAb for 45 min at room
temperature. Sections were incubated with a peroxidase-conjugated goat anti-rat Ig
(Sigma, St. Louis, MO), followed by blocking of free binding sites with 20 % rat
serum for 10 min at room temperature. After staining with 3,3′diaminobenzidine
tetrahydrochloride (Sigma) at a concentration of 0.5 mg/ml in the presence of 0.01
% H₂O₂, sections were incubated with biotin-labeled rat anti-mouse monoclonal
antibody, followed by an incubation with streptavidin. Alkaline phosphatase
activity was visualized using a stock solution of 18.8 mg/ml nitro blue tetrazolium
chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indoly l phosphate toluidine salt,
according to the manufacturer's instructions (Boehringer, Mannheim, Germany).

Quantification of serum immunoglobin levels. Serum was collected from 1 to 6
week-old wild-type and homozygous F/A-2 transgenic littermates. IgG1, IgG2A,
IgG2B, IgG3, IgM and IgA levels were each quantified using ELISA-based assays
according to the manufacturer's instructions (Southern Biotechnology Associates,
Birmingham, AL, USA). 96-Well ELISA plates were prepared by overnight
coating with goat anti-mouse Ig. Plates were blocked for 1 hour at room
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temperature with 1 % BSA in PBS, except for IgA, which was blocked with 3 % non-fat milk powder in PBS. Incubation with serum dilutions in PBS containing 0.05 % Tween-20, pH 7.4 and specific horseradish peroxidase-conjugated, isotype-specific anti-mouse immunoglobulins was for 1 hour at room temperature. Staining was performed using OPD substrate in the presence of H₂O₂. The optical density was read at 405 nm on a microplate reader.

Statistics. Biochemical data on immunoglobulins were tested with a two-way repeated measure analysis of variance (ANOVA; factors age and genotype). Results were considered significantly different at p<0.05. Counts on Peyer's patches were tested with a Poisson distribution.
Chapter  V

Results

Impaired development of Peyer's patches in arginine-deficient transgenic mice.

The phenotype of homozygous F/A-2 transgenic mice (F/A-2⁺/⁺) was described in detail elsewhere (18). The intestinal arginase activity caused a decrease in circulating plasma arginine concentration to 80 μM, as opposed to approx. 250 μM in wild-type suckling and 150 μM in adult mice (18).

A striking abnormality in the F/A-2⁺/⁺ mice was the macroscopical absence of Peyer's patches (PP). Whole mount immunohistochemical staining for intestinal expression of VCAM-1 as a parameter for early PP development (23) revealed that F/A-2⁺/⁺ mice developed a normal number of PP anlagen in the first postnatal week (18). However, subsequent development of PP appeared to be critically dependent on arginine availability, because PP remained underdeveloped in the first 4-6 neonatal weeks in F/A-2⁺/⁺ mice, whereas neonatal arginine injections restored the development of PP back to normal (18). Fig. 1 shows an occasionally found PP in an F/A-2⁺/⁺ small intestine at the age of 3 weeks. These PPs hardly protruded from the serosa, which made them difficult to identify macroscopically. In normal mice, PPs consist of large B cell follicles (Fig. 1A) and smaller T-cell-rich areas on the outside of the follicles (Fig. 1C). The rudimental PPs in F/A-2⁺/⁺ mice showed hypoplasia of the B- and T-cell areas, though B cell follicles were still identifiable (Fig. 1B and D). Both control and F/A-2 B cells were positive for membrane-bound IgM (mIgM) (Fig. 1E and F). The interaction between integrin α4β7 and the mucosal vascular addressin MAdCAM-1 is essential for lymphocyte homing to the PPs (24). MAdCAM-1 was normally expressed on high-endothelial venules within the PPs in both control and F/A-2⁺/⁺ mice (Fig. 1G, H). PPs became macroscopically identifiable in F/A-2⁺/⁺ mice at 6-7 weeks of age, in parallel with the gradual decline in the difference of circulating arginine between control and transgenic mice, demonstrating that the development of PPs in F/A-2⁺/⁺ mice was temporarily suspended, but not abolished.
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Fig. 1 Wild-type and F/A-2^{++} Peyer's patches stained for the presence of B220, IgM, CD3ε, and MAdCAM. Histological examination of small intestines occasionally reveals a small Peyer's patch (PP) in ND21 F/A-2^{++} animals. These PPs (left column) are reduced in size and do not protrude from the serosal surface, compared with PPs from wild-type littermates (right column). Nevertheless, immunohistochemical staining for B220- and IgM-positive B cells (panels A,B, and C,D, respectively), CD3^{+} T-cells (panels E,F) revealed the presence of segregated T- and B cell areas in F/A-2^{++} PPs. Endothelial cells in sinuses lining the PPs normally express MAdCAM-1 (panels G, H). Sections were counterstained with haematoxylin. Bar is 200 μm.
Chapter V

T-cell populations in central and peripheral lymphoid organs

Arginine has been reported to alter T-cell function \textit{in vitro} (25). Therefore, we investigated the development and phenotype of T lymphocytes in F/A-2\(^{+/+}\) transgenic mice in more detail. T cells were isolated from thymus, spleen, cervical lymph nodes, mesenteric lymph nodes, and intestine. In the F/A-2\(^{+/+}\) thymus, absolute cell numbers were decreased, but proportional to the decreased body mass of the transgenic mice (18). The fraction of CD3\(^+\)-positive cells and the distribution of CD4,CD8 double-negative, CD4,CD8 double-positive, CD4 single-positive and CD8 single-positive cells showed no major differences between transgenic and wild-type mice at ND21 (Table 1 and Fig. 2, upper panel). These results indicate that maturation and selection of transgenic thymocytes is not hampered by arginine deficiency.

<table>
<thead>
<tr>
<th>thymocytes</th>
<th>% of cells</th>
<th>fraction of CD4(^+)CD8(^-)(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3(^+)CD4(^+)</td>
<td>11.2</td>
<td>WT 18.1 F/A-2(^{+/+})</td>
</tr>
<tr>
<td>CD3(^+)CD8(^+)</td>
<td>4.4</td>
<td>WT 6.8 F/A-2(^{+/+})</td>
</tr>
<tr>
<td>CD4(^+)CD8(^+)</td>
<td>82.4</td>
<td>100 75.7 100</td>
</tr>
<tr>
<td>CD4(^+)CD8(^-)</td>
<td>11.6</td>
<td>14.1 15.8 20.9</td>
</tr>
<tr>
<td>CD4(^-)CD8(^+)</td>
<td>0.84</td>
<td>1.0 1.3 1.7</td>
</tr>
<tr>
<td>CD4(^-)CD8(^-)</td>
<td>1.8</td>
<td>2.2 2.3 3.0</td>
</tr>
</tbody>
</table>

Table 1: expression of T-cell surface markers on thymocytes of wild-type or F/A-2\(^{+/+}\) mice at 4 weeks of age. Thymocytes from either wild-type or F/A-2\(^{+/+}\) mice, analyzed by flow cytometry for the indicated markers. The percentage of CD4 or CD8 single positive or double negative T cells, compared to CD4 CD8 double positive T cells is similar, indicating that F/A-2\(^{+/+}\) thymocytes mature normally. Percentages shown are representative of three analyses.

Peripheral and mesenteric lymph nodes were easily found in F/A-2\(^{+/+}\) mice. The relative number of CD3\(^+\)-, CD4\(^+\)- and CD8\(^+\)-T cells in those lymph nodes was not different from those in normal mice (Fig. 2, lower panel). Similarly, the relative number of CD3\(^-\)-, CD4\(^-\)- and CD8\(^+\)-T cells present in transgenic intestine was not different from wild-type intestine (Fig. 3A-C). Also, the relative number of intestinal IEL, expressing the gut-homing integrin \(\alpha\(_{\text{IEL}}\)\), was not affected (Fig. 3D).
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Fig 2. Flowcytometric analysis of T lymphocytes from thymus, spleen and lymph nodes of 3 weeks old normal and F/A-2-/- mice. Cells were stained with antibodies to CD4 and CD8, and the percentage of CD4-CD8-double-negative, CD4-CD8-double-positive, CD4-single-positive and CD8-single-positive cells is indicated.
However, the absolute number of T cells that could be recovered from the FA-2+/+ intestinal LPL was lower than that isolated from wild-type intestine (not shown) due to the reduced size of the animals and the PPs.

In the spleen of F/A-2+/+ transgenic mice, CD4-positive and CD8-positive T cells were readily found at three weeks of age, with a normal distribution. (Fig 2, middle panel). However, in line with the reduced intestinal T-cell content, the absolute number of T cells was lower than control mice, also when corrected for the reduced body weight of the transgenic mice (not shown).

**Reduced numbers of B cells in the intestine of arginine-deficient mice**

To investigate the presence of lymphocyte populations expressing the pan B cell marker CD19 and the MHCII antigen I-A, intestinal lymphocytes were isolated, purified free of enterocytes and IELs, and analyzed (Fig. 3E and F). As the transgenic PPs are too small to be identified macroscopically, these B cell rich structures were not excised, but included in the lymphocyte fractions of both wild-type and F/A-2+/+ mice. Figs 3 E and F show an almost complete lack of CD19+ and I-A+B cells in the small intestines of F/A-2+/+ mice during the first four weeks of development. At ND 21, the lymphocyte population in the small intestine of F/A-2+/+ mice contained 5 times fewer CD19+ and I-A+B cells than that of wild-type mice (Table 2). The B cells in the LPL fraction are all located in PPs, as CD19+ or I-A+B cells could not be recovered after excision of PPs from wild-type small intestine prior to homogenization (not shown). Thus, the difference in CD19 and I-A staining between wild-type and F/A-2+/+ mice corroborates the macroscopical observation of the virtual absence of PP in F/A-2+/+ mice.

In order to establish whether the effect of arginine on PP development is mediated by the arginine product nitric oxide (NO), we investigated mice carrying null mutations of either of the three isoforms of the NOS gene (26-28). At three weeks of age, a normal PP development was observed in nNOS-, iNOS-, and eNOS-deficient mice, compared to their appropriate controls (18). In addition, B cell numbers were not decreased in intestinal lymphocyte populations isolated from NOS-deficient mice (Table 2).
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Fig. 3 F/A-2−/− small intestines contain a normal number of T cells, but a reduced number of B cells. The percentage of intestinal lymphocytes, including those from PPs that express the T-cell markers CD3, 4, 8, and αIEL, (Panels A-D) as well as the B cell markers CD19 and MHC-II (Panels E–F), was determined in wild-type (black squares), and F/A-2−/− (gray diamonds), by flow cytometry. The percentage of cells that express T-cell markers is similar in both groups, but the percentage that expresses B cell markers is reduced. The reduction in B cells in F/A-2−/− mice can be fully ascribed to the lack of PPs, as no CD19 and I-A positive cells are found after removal of the PPs (see text).

In OTC-deficient Spf-ash mice, arginine levels are decreased to approx. 120 μM (29, 30), that is, to slightly higher levels than those observed in F/A-2+/− mice. However, in contrast to F/A-2 mice, Spf-ash mice suffer not only from hypoargininemia, but also from hyperammonemia and moderately to severely reduced levels of nearly all other amino acids (30). Nevertheless, Spf-ash mice displayed normal PPs and intestinal B cell numbers (Table 2). These observations
show that mucosal arginine deficiency affects PP and B cell development via a mechanism that is selectively dependent on arginine availability.

<table>
<thead>
<tr>
<th>%</th>
<th>WT (FVB)</th>
<th>F/A-2+/+</th>
<th>WT (C57Bl/6) spf-ash</th>
<th>iNOS−/−</th>
<th>nNOS−/−</th>
<th>eNOS−/−</th>
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<tbody>
<tr>
<td>CD19</td>
<td>17.7 ± 5.6</td>
<td>2.7 ± 0.8</td>
<td></td>
<td>12.9 ± 3.7</td>
<td>14.8 ± 2.2</td>
<td>21.3 ± 5.5</td>
</tr>
<tr>
<td>I-A</td>
<td>20.7 ± 5.2</td>
<td>4.0 ± 1.5</td>
<td></td>
<td>13.8 ± 3.3</td>
<td>17.3 ± 1.9</td>
<td>24.7 ± 5.5</td>
</tr>
</tbody>
</table>

Table 2: expression of B cell surface markers on intestinal lymphocytes of various mice strains at 3 weeks of age. The percentage of B cells is selectively decreased in F/A-2+/+ mice, but not in spf-ash, or NOS-deficient lines, compared to their strain-matched wild types. Data given are the means ± SEM of two separate analyses. Asterisks indicate significant differences from wild type (P < 0.05).

Selective defects of B cell subpopulations in other peripheral lymphoid organs

Peripheral and mesenteric lymph nodes were relatively unaffected in F/A-2+/+ mice. In 3 weeks-old F/A-2+/+ mice, the fraction of lymph node B lymphocytes was reduced from 9% wild-type mice, to 5% in the transgenic mice, with slightly more immature phenotypes (not shown). These differences were most pronounced in 3-weeks old mice and least in adult mice, with 4-weeks old animals being intermediate (a reduction from 13% in 4 weeks-old wild types to 8% in transgenics).

Spleen size was severely reduced in suckling F/A-2+/+ mice (18). As a result, the total number of splenic cells in F/A-2+/+ mice was 10 times less than in controls (Table 3). In order to investigate splenic architecture under these conditions, spleens from 3-week old mice were analyzed immunohistochemically (Fig 4 A-L). In the white pulp of wild-type mice, a wide cuff of B cells, expressing the B cell specific splice variant of CD45 (B220) (Fig. 4A), IgM (Fig. 4C) and, to a lesser extent, IgD (Fig. 4E), surrounded the T-cell-rich periarteriolar lymphatic sheath (PALS) (Fig. 4K). In the transgenic spleen, the area of B220+ or IgM+ B cells surrounding the PALS was approx. 3-fold reduced in diameter, compared to that in wild-type spleen (Fig. 4B, D). Splenic sections of 3-week old mice were also incubated with anti-B220 and anti SER-4 (marginal zone macrophage marker, Fig. 4G and H), anti-B220 and anti-MadCAM-1 (Fig. 4I and J), or anti-CD3 and anti-SER-4 (Fig. 4K and L). Anti-MadCAM-1 antibodies label the sinus-lining cells
of the marginal zone. B cell follicles were clearly identified in wild-type mice as B220-positive (Fig. 4G and I), or CD3-negative (Fig. 4K), areas within the SER-4 (Fig. 4G and K), or MadCAM-1 (Fig. 4I) -positive marginal zone. In F/A-2+/+ mice, B cell follicles were virtually absent (Fig. 4H, J and L). Thus, these data suggest that in F/A-2+/+ mice, the splenic architecture is not disturbed, though the decreased number of B cells in the transgenics impairs a normal B cell follicle formation.

Table 3: Reduced recovery of spleen and bone marrow lymphocytes from 3-weeks old, 4-weeks old, and adult mice.

<table>
<thead>
<tr>
<th>age (ND)</th>
<th>genotype (*10^6 cells)</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>21 spleen</td>
<td>55</td>
</tr>
<tr>
<td>28 bone marrow</td>
<td>5</td>
</tr>
<tr>
<td>ad</td>
<td>46</td>
</tr>
<tr>
<td>21 bone marrow</td>
<td>2</td>
</tr>
<tr>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>ad</td>
<td>3</td>
</tr>
</tbody>
</table>

The differentiation and maturational stage of the F/A-2+/+ splenic B cells was further assessed by flow cytometry (Fig. 5). As already indicated by the immunohistochemical analyses, the fraction of B cells in transgenic spleen was reduced to 65% of that in wild-type controls at 3 (Fig. 5A), and 4 weeks (not shown) of age. The fraction of B cells expressing very low levels of CD21 (receptor for mouse complement factor C3), high levels of mIgM, but no IgD, are recent immigrants from the bone marrow, and are designated type I transitional B cells (31). In transgenics, this fraction is modestly decreased to 31% of the total B cells population in the spleen, compared to 38% in wild-type (Fig. 5B). The fraction of B cells, that expresses high levels of both IgM and CD21 and is positive for IgD (Type II transitional B cells, (31)), is increased to a similar extent, indicating a shift from Type I to Type II transitional B cells in the transgenic spleen (Fig. 5B). The population of mature, resting B cells, that express low levels of IgM, high levels of IgD and intermediate levels of CD21, is similar in F/A-2+/+ and wild-type spleen (Fig. 5B and C). This maturation profile could still be observed at 4 weeks of age (not shown).
Fig. 4 Immunohistochemical analysis of expression of B220, IgM, IgD in the spleen of 3-weeks old wild-type and F/A-2^{+/+} mice. Serial sections of wild-type (left panels) and F/A-2^{+/+} (right panels) were stained for the expression of B220 (panels A,B; G,H; I,J), IgM (panels C,D) or IgD (panels E,F). The B220- and IgM-positive B cell layer surrounding the PALS is reduced to a narrow rim of cells. Splenic marginal zones were visualized by staining marginal zone macrophages sialoadhesin-specific mAb SER-4(G,H and K,L; red), and sinus-lining cells in the marginal zone expressing MadCAM-1 (MECA-
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367)(I,J; red). In the same sections, B220 positive cells are stained blue. Follicles of B220 positive blue staining B cells (G-J) within the borders of the marginal zones (*) are easily distinguishable in wild-types, but are lacking in F/A-2**+. Serial sections were also stained for CD3 (KT3) and the SER-4 antigen (K,L; both red). B cell follicles are marked by CD3-negative areas within the SER-4 positive marginal zone. B220 positive B cells migrating through the PALS (black arrow in G) to form follicles are lacking in F/A-2**+. Bar is 100 μm.

Follicular B cells are CD23-positive. Fig 5D shows that the fraction of IgM-positive, CD23-positive cells was not changed in the spleen of F/A-2**+ mice. Together, these results suggest a reduced output of B cells from the marrow of F/A-2**+ mice resulting in a slow fill of peripheral lymphoid organs, in particular the spleen. The B cells that arrive in the spleen, undergo a normal maturation program, and the architecture of the spleen is not grossly disturbed.

**B cell development in F/A**+** mice is hampered at the pro- to pre-B cell transition.**

The consistently decreased B cell content in peripheral organs prompted us to examine the early B cell maturation in the bone marrow of F/A-2**+ mice in more detail. The total number of cells, recovered from bone marrow was reduced in 3-week old transgenic mice, mostly due to a reduced size of the bones in these mice. After 4 weeks of age, and in adults, normal numbers of bone marrow B cells were recovered from F/A-2**+ mice (Table 3). We studied the differentiation stage of B lymphocytes using staining protocols based on the studies of Hardy et al (32), which allowed the identification of precursor B cells in pro-, pre- and immature B cells. In 3 and 4 -weeks-old mice, a substantial reduction of the fraction of lymphoid cells was seen in F/A-2**+ mice (Fig. 6A and B, top panels), and the differentiation profile of the bone-marrow B cells was perturbed. Cells of the B lineage were identified with antibodies to B220.
Flowcytometric analysis of splenic B cells of 3 weeks old normal and F/A-2+/+ mice. Spleen cells were incubated with combinations of antibodies to IgM, IgD, CD45 (B220), CD21, and CD23. The percentage of the indicated subpopulations are shown, as fraction of the total number of cells (panels A), or as fraction of IgM-positive, B220-positive cells (panels B, C and D). The small squares show schematically the analyzed subpopulations: M, mature; T1, transitional type I; T2, transitional type II, Fol, follicular B cells.
Fig. 6. Flow cytometric analysis of bone marrow B-lineage cells of 3 and 4 weeks old normal and F/A-2 \(^{++}\) mice. Panel A: 3 weeks old mice, panel B, 4-week old mice. B-lineage lymphocytes are contained in the red windows of the upper panels. Cells in this window were analyzed for the expression of IgM and CD45 (B220) (middle panels), and the B220-positive, IgM-negative cells were analyzed for the expression of CD24 and CD43 (S7) (lower panels). The percentage of the indicated subpopulations are shown, as fraction of the total number of cells (upper panels), as fraction of the cells in the B-lineage cell window (middle panels), and as fraction of the B220-positive, IgM-negative cells in the lower panels. The ratio of pre- to pro-B cells is also given in the lower panels. Panel B: 4 weeks old mice. Cells were analyzed as in A), except that for the analysis of the B220-positive, IgM-negative cells antibodies to CD25 and CD43 were used. The small square shows schematically the analyzed subpopulations: M, mature B cells; T1, transitional type I B cells; I, immature B cells; P, pre- and pro-B cells; O, other cell types.
Membrane-IgM-negative, B220-positive cells were further characterized by labeling them with antibodies to CD43 and CD24 (Fig. 6 A, middle and lower panels), or with antibodies to CD43 and CD25 (Fig. 6B, middle and lower panels). The middle panel of Fig. 6A clearly shows that F/A-2\textsuperscript{+/-} mice had a reduced pool of
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pre- and pro-B cells of less than 50% of wild-type controls. This reduction was mainly due to a severe reduction in the fraction of pre-B cells (Figs 6A and B, lower panels). At 3, as well as 4 weeks of age, the ratio of pre- to pro-B cells was reduced from 3.9 and 2.5, respectively, in wild-type mice to 0.7 and 0.8, respectively, in F/A-2/+ animals (Figs 6A and B, lower panels). The subsequent differentiation stages were also affected: immature B cells (low levels of mIgM), type I transitional B cells (high levels of mIgM) and mature, recirculating B cells (lower levels of mIgM, mIgD-positive and high levels of CD45) were all severely reduced down to 10-25% of wild-type fractions. In concert, the fraction of non-B cells was doubled (Figs. 6A and B, middle panels).

Together, we conclude that B cell development in the bone marrow F/A-2/+ mice is impaired at the transition from the pro- to the pre-B cell stage. Hence, a reduced number of B cells leave the bone marrow and enter the periphery. The B cell maturation in the secondary lymphoid organs, however, is not grossly affected.

Decreased serum immunoglobulin M production in F/A-2/+ transgenic mice.

The reduced number of B cells in the spleen and gut of F/A-2/+ mice should have a functional impact on immunoglobulin production. Before weaning, IgG isotypes, but not IgM, are actively taken up by the intestinal epithelium from the mother’s milk by specific Fc-receptors on the brush border of the intestinal epithelium (33). Fig. 7 shows serum immunoglobulin levels in wild-type and transgenic mice from birth through adulthood. IgG concentrations in serum of suckling transgenic mice were not different from those in wild type mice, indicating that the intestinal uptake mechanism was not affected in the transgenics. In contrast, the IgM level was significantly lower (p<0.01) in suckling transgenic mice, while IgA was not yet detectable. After weaning, IgG2A and IgG2b levels increased towards a similar adult level in both wild-type and F/A-2/+ mice. The concentration of IgM also increased, but remained at a significantly lower level in transgenics than in wild-type mice (p<0.05). The levels of IgA and IgG1 tended to increase at a slower pace in transgenic mice, but the differences were not significant.
Chapter V

Fig. 7 Serum immunoglobulin levels in wild-type and F/A-2<sup>+</sup> mice. Serum immunoglobulin levels in wild-type (gray diamonds), and F/A-2<sup>+</sup> (black squares) increase with a similar time course after weaning, but IgM levels are depressed to 30% of control in the F/A-2<sup>+</sup> serum (p<0.05). Furthermore, IgG1 levels do not increase after weaning in the F/A transgenics. For each measurement, serum of three mice was pooled. Values are ± SEM and based on triplicates in two independent analyses.

**B cells of F/A-2<sup>+-/+</sup> mice show normal proliferative responses**

In order to establish whether the hampered development of B cells is due to a defective proliferative capacity, we stimulated primary B cell cultures *in vitro* (Fig. 8). Lymphocytes, isolated from the spleen of 3 week-old wild-type and transgenic mice were stimulated for 2 and 3 days with the polyclonal B cell activator LPS. A comparable proliferative response was seen in B cells, isolated from either F/A-2<sup>+-/+</sup> or wild-type mice. Also in experiments where B cells were stimulated with a goat F(ab')2 anti-μ antibody, no differences in proliferation were found (Fig. 8).
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Fig. 8. In vitro stimulation of splenic B cells of F/A-2 mice elicits a normal proliferation. Splenic lymphocytes (1 * 10^5 cells per well) of 4 weeks old mice were started with goat F(ab') anti-mouse μ chain (αIgM), LPS, IL 4, or anti-CD40 antibody, either alone or in combination for the indicated times. Cells isolated from either wild-type or F/A-2<sup>−/−</sup> spleen displayed a similar proliferative response upon stimulation. Data shown are the means ± SEM of three measurement sessions, each with 3 cultures per condition.

Stimulation of B lymphocytes by T-helper cells in vivo is dependent on the binding of CD40 on the B cell. Culture of B cells in the presence of anti-CD40 antibodies and IL 4 normally results in a strong proliferative response and isotype switching (34). Therefore, B cells were also stimulated with anti-CD40 antibody alone, with IL 4 alone, or anti-CD40 antibody together with IL 4. Fig. 8 shows that none of these challenges revealed a defective transgenic B cell proliferation, compared to wild-type responses. The analysis of culture supernatants for the presence of IgG1 and IgE antibodies revealed normal levels both in cultures of F/A-2<sup>−/−</sup>-derived B cells and in cultures of control B cells (data not shown). These data demonstrate that, despite the observed retardation in B cell maturation, transgenic B cells are
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fully capable of mounting appropriate responses both to T-cell-independent (LPS, anti μ) and T-cell-dependent (CD40/IL-4) stimuli.
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Discussion

At present, limited information is available on how nutrients influence the development of lymphoid organs or immune function. Arginine was surmised to be beneficial for immune system responses (35), but the reported effects are assorted, ranging from an increased production of polyamines (36), via amelioration of tumor growth (37, 38) and wound healing (10, 11), to a direct effect on T-cell gene expression (25). Here, we show that an arginase-mediated selective arginine deficiency results in an obstruction of B cell development in the bone marrow. This is accompanied by a pronounced reduction in the number of peripheral B cells and the macroscopic absence of Peyer's patches. Development of T cells in the thymus appears normal, although the size of the thymus is reduced in proportion to the general growth retardation of arginine-deficient mice (18). However, the number of T cells in the spleen is more reduced than expected. This is not an unusual finding in the spleen of mice with a severe distortion of B cell development (M.C.L., unpublished observations). Peripheral and mesenteric lymph nodes are affected to a much lesser extent. The severity of the phenotype depends on the level of arginase expression in the enterocytes, with hemizygous F/A-2 mice showing an intermediate phenotype. Furthermore, daily arginine injections reverse the lymphocyte phenotype of F/A-2+/− mice (18). The syndrome begins to mitigate after weaning, that is, when intestinal synthesis of arginine ceases and the arginase-expressing enterocytes only catabolize circulating arginine. For these reasons, we believe that this phenotype develops as a result of a selective arginine deficiency.

How can we explain the phenotype? Strikingly, only precursor B lymphocytes appear to be affected. Later steps in development, i.e. immature, transitional and mature B cells in the bone marrow, and the spleen were all reduced in number, but not disproportional. This suggests an unaltered capacity of pre-B cells to mature, once they escape the early maturation block. Further, lack of PP development is associated with B cell deficiency (39, 40). We found no gross defect in T-cell maturation in the thymus, or aberrant T-cell populations in peripheral lymphoid organs. Moreover, T-cell-deficient mice develop normal PPs (41, 42). It therefore does not seem far-fetched to assume that the defective generation of B-
Chapter V

lymphocytes forms the basis of the phenotype. In concert, B cells isolated from peripheral lymphoid organs were able to mount a normal proliferative response upon B cell-specific stimulation, demonstrating that the effect of arginine deficiency on B cell development is restricted to the bone-marrow compartment.

B cells develop in the marrow from hemopoietic precursor cells, and can be divided in several differentiation stages that are characterized by surface phenotype (32, 43). Using the staining protocols of Hardy et al. (32), we found that the relative number of pro-B cells was normal in suckling F/A-2+/+ mice, although the absolute numbers were reduced due to the substantially smaller size of the long bones of the transgenic mice in comparison to the control animals. The population of pre-B cells was severely reduced. What can be the reasons for this developmental block? Early B cell progenitors require cell-to-cell contacts and growth factors, like c-kit ligand and IL7, for further development into pre-B cells (44). The transition of pro- to pre-B cells is marked by the successful completion of the rearrangement process at the heavy chain locus (45). This allows the formation of a premature B cell receptor (BCR) complex with the heavy chain and two surrogate light chains. The constitutive signal that is generated by the expression of this receptor and an IL-7-dependent signal collaborate in the induction of a rapid expansion of the pre-B cell pool (46). The study of mouse strains with induced mutations has revealed several factors that are necessary for the transition of the pro- to pre-B cell stage and the subsequent expansion of the pre-B cell pool. As could be expected, most factors influence the signal transduction pathways initiated by the premature BCR complex or by IL-7. Limited or no expansion of the pre-B cell pool is seen in mice lacking i) components of the BCR complex (e.g. the cytoplasmic domain of the Ig-α chain (47) or the Ig-β chain (48)(signal transduction effectors of the BCR), λ5 (49)(a component of the surrogate light chain of the premature BCR)), ii) components of the signal transduction pathway of the BCR (e.g. syk (50), BLNK (51), vav (52) and PI3K (53)(all signaling intermediates of the BCR)), iii) IL-7 (54) or components of the IL-7 receptor (e.g. IL-7Rα (46), the common γ-chain (55)), or Jak3 (56), and iv) transcriptional activators of components of the BCR or IL-7/IL-7
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receptor system (e.g. E2A (57), Pax-5 (58), EBF (59), Sox-4 (60), mel-18 (61) or C/EBP/β (62)).

Because we assume that an arginine-dependent metabolic mechanism involved in the transition of pro- to pre-B cells is hampered, we do not expect a fully penetrant phenotype, and therefore factors that are known to cause a complete block can be candidate targets as well. The few pre-B cells that escape the block, develop normally. Furthermore, peripheral B cells can be readily induced to differentiate or proliferate by a variety of stimulants mimicking T-cell-dependent and -independent activation. These observations make a B cell autologous defect (i.e. defects in the signaling pathway originating from the (premature) BCR) unlikely.

IL-7 plays a role in both the development of T and B cells. IL-7 deficiency (54, 63), or deletions of components of the IL-7 receptor (46, 54-56) cause a strong reduction in the cellularity of the thymus, with disturbances in the early precursor populations, but with a normal distribution of CD4- or CD8-double-negative, -double-positive, and -single-positive cells (63). Further, a severe deficiency of thymic and peripheral γδ T cells is found (64). We think that the reduced number of thymocytes found in the F/A-2+/+ mice is due to the smaller body size of these mice, but a final conclusion awaits a careful analysis of the precursor T-cell populations. Mice with deletions of transcriptional activators often display a broader phenotype (PAX-5 (58), Sox-4 (60), E2A (57), mel-18 (61)) than that seen in our transgenic mice. We therefore hypothesize that the disturbance in the development of B cells is either caused by a specific defect of genes expressed at the transition of pro- to pre-B cells (e.g. λ5 or V-pre-B, forming the surrogate light chain, or the IL-7 receptor α-chain), or by a bone marrow-specific defect of stroma cells that produce factors interacting with the IL-7 receptor α-chain, i.e IL-7 or thymic stromal lymphopoietin (65).

Mice that lack B cells, suffer from a deficient development of PPs, follicle-associated epithelium and M-cells (39). The near-absence of B cells in the F/A-2+/+ small intestine may therefore suffice to explain the rudimentary development of PPs in these mice. In agreement with this hypothesis, a normal number of VCAM-1-
positive PP anlage was present in F/A-2+/+ intestine in the first postnatal week (18). The follicle-associated epithelial cells and the M-cells, that is, the specialized cells on the luminal surface of the PP dome that are involved in tunneling pathogens from the lumen into the basal surface, also develop under the influence of B-, and not T-lymphocytes (66). Accordingly, the follicle-associated epithelial domes on rudimentary PPs of F/A-2+/+ intestines were reduced in size and the density of M-cells in these domes was less (unpublished observations). However, the PP phenotype may also result from local interference of arginine deficiency with signal-transduction pathways. Recently, it has been reported that transgenic expression of IL-7 in the enterocytes of IL-7-knockout mice reestablished PP development (67), implying that IL-7 biosynthesis in arginine-deficient mice might not only be compromised in bone marrow, but also in enterocytes. VCAM-1-positive PP anlage do not form in absolute IL-7 deficiency, but, as mentioned before, we do not expect a full penetrance of the deficiency in F/A-2+/+ neonates. At present, solving the mechanism underlying the deficient development of PPs in arginine-deficient mice therefore seems more complex than addressing the pre- to pro-B cells obstruction in the bone marrow.

Several reports have suggested that the amino acid arginine is a potential immunostimulant (35, 68), but the molecular and cellular mechanism via which arginine modulates lymphocyte biology and exerts its beneficial effect on mucosal defense, has remained obscure. Under normal conditions, arginine deficiency may not readily occur. However, under certain conditions of malnutrition, as they occur due to a poor diet or old age, a deficiency may occur. It is interesting in this regard, that at older age, bone marrow stromal cells have an impaired ability to support B cell poiesis, most likely due to an impaired release of IL-7 (69). The current study demonstrates an unambiguous effect of arginine deficiency on early B cell development. This finding should allow us to identify the site of action of arginine and to assess the prospects of arginine to become a bona fide immunonutrient.
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References


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Appendix
Appendix I. Circulating and intestinal amino acid levels in wild type (white bars), F/A-1°, and F/A-2° mice of the indicated age. Mice were bled after decapitation at 3 pm. Amino acids were extracted and analysed as described in the materials and methods section in Chapter III. Values are given in μM and mmol/kg tissue for plasma and intestinal tissue respectively. Each bar is a mean ± SEM of 6-10 mice. Male and female mice were pooled.
Appendix I. Continued
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arginine

\[
\begin{align*}
\text{HO-C=O} & \\
\text{H}_2\text{N-C-H} & \\
\text{(CH}_2\text{)}_3 & \\
\text{NH} & \\
\text{H}_2\text{N-C=NH} & \\
\end{align*}
\]

ornithine

\[
\begin{align*}
\text{HO-C=O} & \\
\text{H}_2\text{N-C-H} & \\
\text{(CH}_2\text{)}_3 & \\
\text{NH} & \\
\text{H}_2\text{N-C=NH} & \\
\end{align*}
\]

glycine

\[
\begin{align*}
\text{HO-C=O} & \\
\text{H}_2\text{N-C-H} & \\
\text{(CH}_2\text{)}_3 & \\
\text{NH} & \\
\text{H}_2\text{N-C=NH} & \\
\end{align*}
\]

argininosuccinic acid

\[
\begin{align*}
\text{HO-C=O} & \\
\text{H}_2\text{N-C-H} & \\
\text{(CH}_2\text{)}_3 & \\
\text{NH} & \\
\text{C=NH} & \\
\text{NH} & \\
\text{HC-C=O} & \\
\text{H}_2\text{C-OH} & \\
\text{HO-C=O} & \\
\end{align*}
\]

guanidinosuccinic acid

\[
\begin{align*}
\text{HO-C=O} & \\
\text{H}_2\text{N-C-H} & \\
\text{(CH}_2\text{)}_3 & \\
\text{NH} & \\
\text{C-}\text{C=O} & \\
\text{H}_2\text{N-C=NH} & \\
\end{align*}
\]

agmatine

\[
\begin{align*}
\text{HO-C=O} & \\
\text{H}_2\text{N-CH}_2 & \\
\text{(CH}_2\text{)}_3 & \\
\text{NH} & \\
\text{H}_2\text{N-C=NH} & \\
\end{align*}
\]

\[\alpha\text{-N-acetylarginine} \quad \alpha\text{-NAA}\]

\[
\begin{align*}
\text{H}_3\text{C-C-N-C-C=O} & \\
\text{(CH}_2\text{)}_3 & \\
\text{NH} & \\
\text{H}_2\text{N-C=NH} & \\
\end{align*}
\]

Homoarginine

\[
\begin{align*}
\text{HO-C=O} & \\
\text{H}_2\text{N-C-H} & \\
\text{(CH}_2\text{)}_3 & \\
\text{CH}_2 & \\
\text{NH} & \\
\text{H}_2\text{N-C=NH} & \\
\end{align*}
\]

Appendix II. Structural formulas of guanidino compounds
<table>
<thead>
<tr>
<th>Amino acid levels and structural formulas</th>
</tr>
</thead>
<tbody>
<tr>
<td>guanidino acetate</td>
</tr>
<tr>
<td>GAA</td>
</tr>
<tr>
<td>HO-C=O</td>
</tr>
<tr>
<td>C=H</td>
</tr>
<tr>
<td>N-H</td>
</tr>
<tr>
<td>H$_2$N-C=NH</td>
</tr>
<tr>
<td>creatine</td>
</tr>
<tr>
<td>CT</td>
</tr>
<tr>
<td>HO-C=O</td>
</tr>
<tr>
<td>C=H</td>
</tr>
<tr>
<td>N-C=H</td>
</tr>
<tr>
<td>H$_2$N-C=NH</td>
</tr>
<tr>
<td>methylguanidine</td>
</tr>
<tr>
<td>MG</td>
</tr>
<tr>
<td>C=H$_3$</td>
</tr>
<tr>
<td>N-H</td>
</tr>
<tr>
<td>H$_2$N-C=NH</td>
</tr>
<tr>
<td>creatinine</td>
</tr>
<tr>
<td>CTN</td>
</tr>
<tr>
<td>HO-C=O</td>
</tr>
<tr>
<td>C=H</td>
</tr>
<tr>
<td>N-C=H</td>
</tr>
<tr>
<td>H$_2$N-C=NH</td>
</tr>
<tr>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GABA</td>
</tr>
<tr>
<td>HN---C=O</td>
</tr>
<tr>
<td>HN=Ca</td>
</tr>
<tr>
<td>N=CH$_2$</td>
</tr>
<tr>
<td>CH$_3$</td>
</tr>
<tr>
<td>β-guanidino proprionic acid</td>
</tr>
<tr>
<td>β-GPA</td>
</tr>
<tr>
<td>HO-C=O</td>
</tr>
<tr>
<td>(CH$_2$)$_2$</td>
</tr>
<tr>
<td>NH</td>
</tr>
<tr>
<td>H$_2$N-C=NH</td>
</tr>
<tr>
<td>γ-guanidinobutyric acid</td>
</tr>
<tr>
<td>γ-GBA</td>
</tr>
<tr>
<td>HO-C=O</td>
</tr>
<tr>
<td>(CH$_2$)$_3$</td>
</tr>
<tr>
<td>NH</td>
</tr>
<tr>
<td>H$_2$N-C=NH</td>
</tr>
<tr>
<td>δ-guanidinovaleric acid</td>
</tr>
<tr>
<td>δ-GVA</td>
</tr>
<tr>
<td>HO-C=O</td>
</tr>
<tr>
<td>(CH$_2$)$_4$</td>
</tr>
<tr>
<td>NH</td>
</tr>
<tr>
<td>H$_2$N-C=NH</td>
</tr>
</tbody>
</table>

Appendix II. Structural formulas continued
Chapter VI

\[ \text{α–keto-δ-guanidinovaleric acid} \quad \text{argininic acid} \]

\[ \text{α–K–δ–GVA} \quad \text{ArgA} \]

\[ \begin{align*}
&\text{HO-C}=\text{O} \\
&\quad \text{C}=\text{O} \\
&\quad (\text{CH}_2)_3 \\
&\quad \text{NH} \\
&\text{H}_2\text{N}-\text{C}=\text{NH} \\
&\quad \text{HO-C}=\text{O} \\
&\quad \text{C}-\text{OH} \\
&\quad (\text{CH}_2)_3 \\
&\quad \text{NH} \\
&\text{H}_2\text{N}-\text{C}=\text{NH}
\end{align*} \]

Appendix II. Structural formulas continued
Samenvatting

In dit proefschrift staat het aminozuur arginine centraal. Arginine is precursor voor de productie van een scala aan fysiologisch zeer belangrijke moleculen, waaronder creatine en stikstofoxide, maar bovenal is arginine een intermediair van de ornithine cyclus. Deze cascade van enzymreacties heeft ten doel het omzetten van het giftige ammoniak, afkomstig van eiwitafbraak, in ureum, dat via de urine kan worden uitgescheiden. Hiertoe wordt eerst ammoniak en koolstofdioxide omgezet in carbamoylfosfaat en citrulline. Deze omzettingen vinden plaats in het mitochondrion van de cel. Vervolgens wordt dit citrulline in het cytoplasma omgezet in arginine, waarna ureum wordt afgesplitst door het enzym arginase, waarbij ornithine ontstaat, wat weer in de ornithine cyclus kan worden opgenomen. Dit proces van ureum vorming vindt in zijn geheel plaats in de lever.

Arginine is een belangrijk precursor molecuul, wat in aanvulling op het dieet dient te worden synthetiseerd. Het wordt aangeduid als zijnde conditioneel essentieel; het kan worden aangemaakt, maar in omstandigheden waarin meer arginine wordt gevraagd, zoals groei, of in pathologische condities, kan de geproduceerde hoeveelheid arginine te beperkt zijn. Van de lever is bekend dat het niet bijdraagt aan het netto afgifte van arginine aan de circulatie, vanwege de grote hoeveelheid hepatisch arginase. In Hoofdstuk II is de rol van de dunne darm in de arginine productie nader bestudeerd in de rat, door analyse van de expressie van ornithine cyclus enzymen in het darmepitheel gedurende pre- en postnatale ontwikkeling. De mitochondriale ornithine cyclus enzymen worden continue tot expressie gebracht in de enterocyten van de crypten en aan de basis van de villi, zodat citrulline biosynthese aldaar mogelijk is. Daarnaast komen, in de peri- en postnatale periode, de cytosolair enzymen tot expressie in de enterocyten aan de villus toppen, wat de omzetting van citrulline in arginine mogelijk maakt in dit compartiment. Op latere leeftijd, als de levensduur van enterocyten wordt verkort, wordt de expressie van de cytosolair enzymen niet meer waargenomen, terwijl tevens arginase tot expressie komt. De volwassen darm is dus niet meer in staat arginine te produceren. De expressie van ornithine cyclus enzymen, betrokken bij
de biosynthese van arginine, lijkt zich aldus aan te passen aan een periode van snelle groei, en hoge arginine vraag.

De fysiologische gevolgen van een chronisch arginine tekort, geïnitieerd ofwel door een arginine-arm dieet, een defect in productie, of door een verhoogde vraag, kondig nooit goed in kaart gebracht worden, omdat een geschikt diermodel ontbrak. Daartoe zijn transgene muizenlijnen gegenereerd, waarin arginase tot overexpressie wordt gebracht in de enterocyten van het villus compartiment waar normaliter arginine wordt gemaakt. De karakteristieke fenotypen van twee lijnen van transgenen met een verschillende mate van expressie van arginase worden beschreven in *Hoofdstuk III*. De transgenen (F/A muizen) vertonen een gegradeerde argininespiegel verlaging, afhankelijk van de mate van de expressie van arginase. Deze verlaging resulteert in een groeiachterstand en slechte ontwikkeling van de spieren en vacht. Dit fenotype kan niet zondermeer worden verklaard door gebrekkige eiwitsynthese door tekort aan arginine, aangezien een arginine-rijk eiwit als trychohyaline, wat in haarfollikels voorkomt, normaal tot expressie komt. De ontwikkeling van dit fenotype was met arginine, maar niet met creatine suppletie tegen te gaan, al vertonen de F/A muizen naast een arginine, tevens een creatine verlaging. Ook in volwassen transgenen wordt nog een verlaagde argininespiegel gemeten, wat, gezien het verdwijnen van de arginine biosynthetiserende capaciteit uit de darm na het spenen, aangeeft dat de transgene darm veel arginine uit de circulatie opneemt.

Arginine wordt gekenmerkt door de aanwezigheid de zeer stikstofrijke "guanidino groep", welke in vele metabolieten van arginine, de guanidino verbindingen, is terug te vinden. Sommige van deze verbindingen, zoals creatine en creatinine, hebben een belangrijke fysiologische functie, terwijl andere, zoals guanidinosuccinaat (GSA), (neuro)toxisch zijn. Bij nierdeficiëntie en condities waarbij de ornithine cyclus verstoord is, zoals metabole ziekten, worden veranderde spiegels van deze guanidino-derivaten, met name een verhoogde GSA spiegel, gedacht bij te dragen aan de (neuro)pathologie. Het metabolisme van deze guanidino-derivaten is nog op belangrijke punten onbegrepen. De specificiteit van het arginine-deficiënte F/A muizenmodel maakt een analyse van de rol van arginine
in dit metabolisme mogelijk. In Hoofdstuk IV wordt beschreven dat arginine deficiëntie is geassocieerd met een verlaagde spiegel van alle guanidino-derivaten van arginine, behalve GSA, dat juist verhoogd is. Deze veranderingen zijn afhankelijk van de beschikbaarheid van arginine, aangezien arginine suppletie de spiegels in de transgenen normaliseert. De verhoging van de GSA spiegels gaat gepaard met een verminderde prestaties in gedragstesten voor neuromotor functies, wat de suggestie van neurotoxiciteit versterkt.

Arginine wordt wel een immunonutriënt genoemd, vanwege een vermeende positieve werking op de immuunrespons in klinische setting. Aldus worden grote hoeveelheden arginine aan perioperatieve voedingssupplementen toegevoegd, alhoewel een moleculaire verklaring voor dit effect ontbreekt. Een aanwijzing die een positieve invloed van de aanwezigheid van arginine op de leukocyt functie bevestigt, is dat één van de kenmerken van de arginine deficiënte F/A muizen het verminderd tot ontwikkeling komen van de platen van Peyer, de lymfoïde folliculaire organen in de darm, is. Verder hebben jonge F/A muizen een verminderd miltgewicht en verminderde immunoglobuline M productie, terwijl de thymus zich normaal ontwikkelt. In muizen, deficiënt voor het enzym stikstofoxide (NO) synthase worden deze afwijkingen niet waargenomen, wat wijst op een werkingsmechanisme dat NO onafhankelijk is. In Hoofdstuk V volgt een verdere analyse van platen van Peyer, milt, en lymfklieren. Cellulaire bestudering van deze perifere lymfoïde organen onthulde een verminderd aantal rijpe B-cellen, terwijl T-cel populaties normaal zijn. Aan de hand van bestudering van vroege rippingsstadia van B-cellen in het beenmerg werd vastgesteld dat de rijping van pro- naar pre B-cel is verstoord in F/A muizen, waardoor een verminderd aantal rijpe B-cellen het beenmerg kunnen verlaten. Deze observatie verklaart de afwijkingen in de lymfoïde organen in de arginine deficiënte muizen. Momenteel wordt gezocht naar mogelijke verstoringen in de expressie van moleculen die op de B-cel rijping in het beenmerg van invloed zijn.
Publicaties


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Paradoxaal genoeg krijgt dat deel van het proefschrift met de hoogste impact factor gewoonlijk de minste aandacht van de promovendus. Ik sluit me volledig aan bij dit gebruik door een dag voor het ter perse gaan van dit proefschrift aan het dankwoord te beginnen. Een aantal mensen moet ik in het bijzonder bedanken. Om te beginnen mijn hooggewaardeerde promotor Wout Lamers, en “trans-acting” promotor-op-afstand Peter Soeters. Wout, jouw tomeloze inzet, enthousiasme en ideeën heb ik enorm gewaardeerd. Ik ben blij dat je streven eens samen met je broertje te publiceren in “een fatsoenlijke krant” nu bereikt is. Na de bezoeken aan broer Rinus in Freiburg blijkt Wout de toewijding aan de wetenschap niet van een vreemde te hebben.

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Curriculum Vitae
