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18. After the animal was given an overdose of pentobarbital, the brain was removed and transected through the upper mesencephalon. One hemisphere was immersed in 4 percent formaldehyde solution and processed for paraffin embedding; the other hemisphere was cut coronally into 1-cm-thick slabs and then frozen individually in liquid nitrogen (−70°C).
19. S. Tsuji, *Histochemistry* **42**, 99 (1974), with half the suggested substrate concentration (acetothiocholine iodide); the product was precipitated with 3 percent potassium ferricyanide. Congo red staining was performed according to *Manual of Histological Staining Methods of the Armed Forces Institute of Pathology*, L. C. Luna, Ed. (McGraw-Hill, New York, ed. 3, 1973). AChE specificity was supported by controls: ethopropazine ($10^{-4}M$) in the incubation medium inhibited pseudocholinesterases; AChE was inhibited by eserine sulfate ($10^{-4}M$); and nonenzymatic precipitation was used to control for absence of substrate. Ethopropazine had no effect on the staining of plaques; the absence of substrate or the inclusion of eserine sulfate in the incubation medium eliminated plaque staining.
20. Plaques were characterized as immature, mature, and end-stage amyloid-rich, depending on their content of neurites and amyloid. They were counted by three independent observers; scores are averages of three counts. The areas counted were computed using a Hewlett-Packard digitizer.
21. Fixed sections were stained with cresyl violet, hematoxylin and eosin, Luxol fast blue, Congo red and hematoxylin, and with silver by the Sevier-Munger silver method.
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Systemic Lupus Erythematosus-Like Syndrome in Monkeys Fed Alfalfa Sprouts: Role of a Nonprotein Amino Acid

Abstract. *Hematologic and serologic abnormalities similar to those observed in human systemic lupus erythematosus (SLE) developed in cynomolgus macaques fed alfalfa sprouts. L-Canavanine sulfate, a constituent of alfalfa sprouts, was incorporated into the diet and reactivated the syndrome in monkeys in which an SLE-like syndrome had previously been induced by the ingestion of alfalfa seeds or sprouts.*

Systemic lupus erythematosus (SLE) has been observed in monkeys fed alfalfa seeds (*1*, *2*). This primate model of SLE is characterized by an anemia that results from the occurrence of antibodies to red blood cells, by lowered complement components in the serum, by antibodies to nuclear antigens (ANA), antibodies to double-stranded DNA (anti-dsDNA), lupus erythematosus (LE) cells in peripheral blood smears, and the deposition of immunoglobulin and complement in the kidneys and skin (*2*). We have proposed the hypothesis that this syndrome may be triggered by L-canavanine found in alfalfa seeds (*3*). L-Canavanine, $H_2N\text{-}C(=NH)\text{-}NH\text{-}O\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)COOH$, is the guanidinoxy structural analog of arginine; it constitutes about 1.5 percent of the dry weight of alfalfa seeds and alfalfa sprouts (*4*) and is toxic to many organisms (*5*), including mammals (*6*).

Twelve adult female cynomolgus macaques (*Macaca fascicularis*), obtained in Indonesia, were randomly assigned to two groups of six animals each and fed, for 7 months, semipurified food (group 1) or similar food containing 40 percent (oven-dried) alfalfa sprouts (group 2). The percentage composition of semipurified food (group 1) was: casein, 18; sugar, 30; Alphacel, 12; honey, 10; banana, 10; butter, 3; coconut oil, 8.5; safflower oil, 2.5; cholesterol, 0.108; and additional vitamins and salts. The amount of 40 percent dried alfalfa sprouts added to the diet of animals in group 2 was based on isocaloric substitution of nutrients; that is, the percentage composition of oven-dried alfalfa sprouts was: moisture, 10; protein, 31.2; fat, 2.08; carbohydrate, 49.4; fiber, 16.1; and ash, 7.3. The caloric value was 270 kcal/100 g (*7*). Group 2 continued the alfalfa sprout diet for an additional 3 months. Venous blood was obtained before dietary manipulation and at monthly intervals thereafter. The following determinations were made: complete blood count and Technicon Sequential Multiple Analyzer Computer Chemistry Screen (SMAC); ANA test by indirect immunofluorescence with the use of 4-μm sections of frozen mouse kidney and the HEp-2 human cell line (Microbiological Associates) (*8*, *9*) as substrates; anti-dsDNA (*10*); C3 and C4 components of complement (*11*); direct antiglobulin test (Coombs' test) for the determination of antibodies to red blood cells (*12*, *13*).

Significant differences among animals ingesting food with or without alfalfa sprouts were not apparent for the following variables: body weight, food intake, glucose, blood urea nitrogen, creatinine, sodium, potassium, chloride, carbon dioxide, uric acid, calcium, phosphorus, total protein, albumin, triglycerides, total and direct bilirubin, alkaline phosphatase, lactate dehydrogenase (LDH), serum glutamate oxaloacetic transaminase (E.C. 2.6.1.1) and γ-glutamyl transpeptidase (E.C. 2.3.2.2). Plasma cholesterol values (averaged for the first 6 months of the diet) were 395 ± 101 mg/dl (mean ± S.D.) in monkeys on diet 1 (controls) and 186 ± 35 mg/dl in animals on the diet containing alfalfa sprouts ($P < .01$, Student's *t*-test). Additional hematologic and serologic values are shown in Table 1. Control animals and four monkeys ingesting alfalfa sprouts did not show hematologic or serologic abnormalities

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during the observation period, except for the one monkey in group 2 that developed an ANA titer of 1:240. Significant abnormalities appeared in the remaining two animals from group 2. One monkey (10661) developed the antibody-mediated mild anemia after 10 months (lowest hemoglobin, 10.8 g/dl; erythrocyte count, 5.78 × 10^6/mm^3; and hematocrit, 32 percent). These values are low—that is, less than the mean minus 2 S.D. of the controls—and are also generally lower than most determinations performed in more than 500 *M. fascicularis* (*14*). Another group 2 monkey (10670) had hematologic and serologic abnormalities characteristic of SLE after 2 months, that is, anemia (lowest hemoglobin level, 7.5 g/dl; erythrocyte count, 3.32 × 10^6/mm^3; hematocrit, 23.3 percent); ANA 1:240 (rim pattern); anti-dsDNA binding of 21.9 percent; lowered serum complement (C3 component, 92 mg/dl, and C4 component, < 7 mg/dl).

Since the lupus-like syndrome occurs only in a select number of monkeys given alfalfa seeds (*2*) or sprouts, we studied three animals that had previously developed the syndrome but had returned to normal after receiving Purina Monkey Chow diet. Monkey 10670 (see above) was tested 7 months after its return to the Monkey Chow. Monkey 9880 had an ANA titer of 1:480 (rim pattern) after 2 months on the alfalfa seeds diet, with anti-dsDNA binding of 6.6 percent; after an additional 2 months feeding on the alfalfa sprouts diet, the serum complement C4 component was 11 mg/dl; it was tested 13 months after being fed Purina Monkey Chow. Monkey 9704 had an ANA titer of 1:480 (homogeneous pattern) and anti-dsDNA binding of 9.6 percent after 2 months on the alfalfa sprouts diet; it was tested 12 months after being fed Purina Monkey Chow. These three animals were fed 1 percent L-canavanine sulfate (Sigma) in a semipurified diet with components similar to the food described above [caloric content (percent): protein, 12.8; fat, 35.4; carbohydrate, 51.8]. The protein content was decreased to avoid dilution of the foreign amino acid. Blood was drawn weekly for the analyses (Table 2).

In monkey 9880, antiglobulin-positive anemia (lowest hemoglobin level, 10.8 g/dl; erythrocyte count, 5.23 × 10^6/mm^3; hematocrit, 32.3 percent) developed 4 weeks after initiation of the L-canavanine diet. Reduced C3 in the serum was also observed. Monkey 9704 showed profound anemia (hemoglobin level, 7.9 g/dl; erythrocyte count, 4.25 × 10^6/mm^3; and hematocrit, 23.8 percent); the ANA test was abnormal, and lowered serum complement was also observed. This monkey died after 10 weeks of L-canavanine ingestion (L-canavanine sulfate at 2 percent was given during the final 3 weeks). Postmortem examination revealed extensive lobar pneumonia. Monkey 10670 showed anemia 4 weeks after beginning the L-canavanine regimen (hemoglobin, 5.3 g/dl; erythrocyte count, 2.13 × 10^6/mm^3; and hematocrit, 15.5 percent); leukocytosis (34,000 cell/mm^3), abnormal ANA tests, circulating antibody to dsDNA, lowered serum complement, and enlarged spleen and peripheral lymph nodes were observed.

Our data demonstrate that alfalfa sprouts can induce hematologic and serologic abnormalities characteristic of an SLE-like syndrome similar to that noted in monkeys ingesting alfalfa seeds (*1*, *2*). Examination of the monkeys also showed granular deposition of immunoglobulin G and complement at the dermal-epidermal junction and immune-complex–induced glomerulonephritis (*2*). Since not all animals are equally susceptible, the possibility of a genetically mediated mechanism should be entertained. Our findings suggest that L-canavanine—which occurs in relatively large concentrations in alfalfa seeds and sprouts (*4*)—may be involved in the pathogenesis of the SLE-like syndrome since this amino acid induced certain hematologic and serologic abnormalities characteristic of the syndrome. However, a direct toxic effect of L-canavanine (*5*, *6*) or of other components of alfalfa sprouts cannot be excluded. It is unlikely that the lower content of protein in the diet is responsible for this effect; similar amounts of protein have been found adequate to sustain growth in monkeys (*15*). The decrease in blood cholesterol may be an independent effect because saponins are present in alfalfa seeds (*16*). Although L-canavanine is metabolized to urea and guanidine in rats (*17*), the proportion of L-canavanine that can remain intact in monkeys is unknown. Because of structural analogies, L-canavanine may substitute arginine in histones and thus affect the interaction with nucleic acids

Table 1. Hematologic and serologic observations in cynomolgus macaques maintained on a control diet (group 1) or a diet containing alfalfa sprouts (group 2) (mean ± S.D.).

Animals (No.)	Diet (months)	Hemoglobin (g/dl)	Red blood cells (10^6/mm^3)	Hematocrit (%)	Direct antiglobulin test	ANA titer (pattern)	Anti-dsDNA (% binding)	Complement (mg/dl)	
								C3	C4
Group 1, control diet									
6	7	12.9 ± 0.7	6.53 ± 0.41	40.1 ± 2.3	−	−	0.9 ± 0.3	317 ± 34	28 ± 6
Group 2, 40 percent dried alfalfa sprouts									
4	10	13.2 ± 1.3	6.50 ± 0.70	40.0 ± 4.3	−	−*	0.8 ± 0.1	280 ± 38	35 ± 5
1 (10661)	10	10.8	5.78	32	+	−	0.6	247	30
1 (10670)	2	7.5	3.32	23.3	+	1:240†	21.9	92	< 7

*One animal on five occasions had ANA titers between 1:30 (speckled pattern) and 1:240 (homogeneous pattern). †Rim pattern.

Table 2. Hematologic (lowest values) and serologic (most abnormal value) observations in cynomolgus macaques maintained on a diet containing 1.0 percent L-canavanine sulfate. Parentheses indicate initial values.

Monkey number	Diet (weeks)	Hemoglobin (g/dl)	Red blood cells (10^6/mm^3)	Hematocrit (%)	Coombs' test	ANA titer (pattern)	Anti-dsDNA (% binding)	Complement (mg/dl)	
								C3	C4
9880	4	10.8	5.23	32.3	(−) +	(−) −	(0) 2.4	(355) 190	(42) 20
9704	9	7.9	4.25	23.8	(−) −	(−) 1:120*	(0.1) 6.5	(340) 188	(48) 12
10670	4	5.3	2.13	15.5	(−) +	(−) 1:240*	(1.0) 9.2	(324) 72	(29) 7

*Homogeneous pattern.

and the functions of the genome (*18*). Moreover, arginyl–transfer RNA synthetase (E.C. 6.1.1.19) reacts with (charges) L-canavanine (*19*), which is incorporated into proteins (*20*). It has been suggested that the erroneous protein affects synthesis of messenger RNA molecules and disrupts replication (*5*), and it seems possible that the immune system reacts to abnormal canavanyl proteins by giving rise to the autoantibodies seen in this SLE-like syndrome. Canavanine may also function in enzymatic reactions involving arginine as the preferred substrate (*21*). Finally, an additional mechanism for the analog toxicity may result from the hydrolytic cleavage of L-canavanine by arginase to yield L-canaline, H_2N-O-CH_2-CH_2-CH(NH_2)COOH, and urea. Structurally, L-canaline is an analog of ornithine [H_2N-CH_2-CH_2-CH_2-CH(NH_2)COOH]. Antimetabolic properties may result from L-canaline binding with pyridoxal phosphate and the subsequent inactivation of enzymes that require the B_6 cofactor (*22*).

L-Canavanine is one of the numerous nonprotein amino acids with functional similarities to the components of mammalian proteins (*23*) that occur in higher plants (*24*). Whether they may be ingested and thus induce or reactivate SLE in susceptible humans remains to be explored.

M. RENÉ MALINOW
Cardiovascular Diseases, Oregon Regional Primate Research Center, Beaverton 97006

EMIL J. BARDANA, JR.
BERNARD PIROFSKY
SHIRLEY CRAIG
Department of Immunology, Oregon Health Sciences University, Portland 97201

PHYLLIS MCLAUGHLIN
Cardiovascular Diseases, Oregon Regional Primate Research Center

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Calcium and Age in Fibroblasts from Control Subjects and Patients with Cystic Fibrosis

Abstract. *Intracellular calcium increases significantly as human fibroblasts age in culture. The calcium increase occurs 5 to 6 weeks (passages) earlier and is significantly greater in fibroblasts from subjects with cystic fibrosis in comparison with cells from control subjects. Intracellular calcium, which is thought to be a pathogenetic factor in cystic fibrosis, may also be a meaningful marker in cell aging.*

For several decades, sporadic reports of an association between cellular calcium and aging have appeared (*1*). To our knowledge, such a relation has not been described in cultured fibroblasts (*2*), which are used as a model of aging. Because the concentration of intracellular calcium is greater in cells from individuals with cystic fibrosis (CF) (*3*) than in cells from control subjects (*4, 5*) and since cells from individuals with CF appear to age prematurely (*6*), we designed experiments to examine the relation between intracellular calcium and passage number (weekly age in culture) in fibroblasts from control subjects and from patients with CF. We report here results that (i) confirm that increased intracellular calcium occurs in cells from patients with CF in comparison with age- and

Table 1. Description of fibroblast strains used in assays of cellular calcium by atomic absorption.

Fibroblast strain number	Sex	Age (years)	Passage at which calcium was assayed	
			Experiment 1	Experiment 2
		Cells from patients with CF		
29	F	12	15	
44	M	10	15	
45	F	7	15	
46	M	16	6, 7, 15	
55	F	24	15	5 to 20
63	F	(3 months)		4 to 20
69	M	17	6, 7	5 to 20
70	M	21	6, 7	3 to 20
71	M	7	6, 7	5 to 7
72	F	20	6, 7	3 to 20
		Cells from control subjects		
31	F	13	15	
32	M	13	15	
57	F	25	15	
59	M	13	15	
61	F	8	15	
82	F	30		4 to 20
83	F	22	6, 7	5 to 6
84	M	20	6, 7	
85	M	6	6, 7	3 to 20
90	M	20	6, 7	3 to 20
91	M	20	6, 7	4 to 6
92	F	(3½ months)		3 to 20
93	F	(5 months)		
94	F	(5 months)		

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