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Construction of Synthetic Immunogen: Use of New T-Helper Epitope on Malaria Circumsporozoite Protein

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The circumsporozoite (CS) protein of *Plasmodium falciparum* is the focus of intense efforts to develop an antisporozoite malaria vaccine. Localization of sites for T-cell recognition on this molecule is critical for vaccine design. By using an algorithm designed to predict T-cell sites and a large panel of H-2 congenic mice, a major nonrepetitive T-cell site was located. When a synthetic peptide corresponding to this site was covalently linked to the major B-cell site on the molecule, an immunogen capable of eliciting a high-titer antibody response was formed. This peptide sequence could prime helper T cells for a secondary response to the intact CS protein. The new helper T-cell site is located outside the repetitive region of the CS protein and appears to be the immunodominant T site on the molecule. This approach should be useful in the rational design and construction of vaccines.

MUCH EFFORT IS CURRENTLY being devoted to vaccine development as a possible means of preventing malaria. The malaria parasite (*Plasmodium* species) is carried by mosquitoes that inoculate the mammalian host with sporozoites. The sporozoites travel to the liver and commence the exoerythrocytic stage of their life cycle. If they could be blocked before they entered hepatocytes, or if the infected hepatocytes could be destroyed prior to rupture and liberation of merozoites, the disease would be prevented.

Sporozoite-blocking vaccines currently being tested in humans consist of a malarial repeated epitope of the circumsporozoite (CS) protein covalently linked to a sequence unrelated to the parasite. This construction is based on the observations that (i) the central third of the CS protein contains a tandemly repeated epitope that does not differ among the various isolates tested (*1, 2*) and (ii) antibodies to this epitope can prevent invasion of hepatocytes in vitro (*3, 4*) and protect mice in vivo from challenge with murine malaria (*P. berghei*) (*5*). We recently examined the immune response in mice to one of these vaccines, which is referred to as $R32tet_{32}$. It is produced as a fusion protein between 32 tandem repeats $[(NANP)_{15}NVDP]_2$ (single-letter code for amino acid residues; see Fig. 1) derived from the CS protein and part of a sequence (32 residues) encoded by a tetracycline resistance gene read out of frame (*3*). We showed (*6*) that only mice carrying the $I\text{-}A^b$ gene could produce a T-cell response to the malaria-encoded sequence $(NANP)_n$, a finding that has been confirmed by others (*7*). In such mice, $(NANP)_n$ stimulated proliferating T cells as well as helper T cells. The tet_{32} peptide also contained a T-cell site (or sites) but was recognized after immunization with $R32tet_{32}$ by only two of seven congenic mouse strains that differed only at their H-2 loci. If a similar situation occurred in humans, some would not respond to the vaccine. Furthermore, in those humans that did respond, natural boosting from sporozoites would occur only in that subset that responded to $(NANP)_n$, not those who responded only to tet_{32}. Natural boosting is important if constant high levels of antibody are required for protection. A response to a malaria-encoded T-cell epitope is also critical if antibody-independent T-cell immunity is required for protection (*8*).

We therefore searched for other T-cell epitopes on the CS protein of *P. falciparum* that could be included with $(NANP)_n$ in a vaccine for humans. We observed that the immune response to the entire CS protein in mice was under *Ir* gene control. Simultaneously, we analyzed the sequence of the CS protein using an algorithm for predicting T-cell sites (*9, 10*) and found that a potential major site occurred in a region located about 40 amino acids from the tandem repeats, toward the carboxyl terminus. A peptide corresponding to this region elicited an immune response in the same congenic strains that gave the optimal response to the entire CS protein [B10.BR and B10.A(4R)]. We confirmed that this peptide was a helper T-cell site by covalently linking it to the sequence, $NP(NANP)_5NA$. This construct elicited high titers of antibodies in these congenic strains [B10.BR and B10.A(4R)] that did not respond to the repeated tetrapeptide sequence alone.

To determine whether there were T-cell sites other than the NANP repeat on the CS protein, we immunized various congenic strains of mice with an infectious recombinant vaccinia virus encoding the entire CS protein from the 7G8 strain of *P. falciparum* (*1*), and estimated the antibody response to $(NANP)_n$. Because there was no source of purified CS protein, a recombinant vaccinia virus was used for these studies. This recombinant is referred to as CS–vaccinia virus (*11*). Our finding (see Table 1) that the B10 ($H\text{-}2^b$) strain responded to CS–vaccinia virus was expected, since mice carrying the $I\text{-}A^b$ gene have been shown to make antibodies to $(NANP)_n$ after immunization with $(NANP)_n$ (*6, 7*); however, B10.BR and B10.A(4R) mice, whose T cells do not respond to $(NANP)_n$, also produced antibodies to the repeat sequence. The other strains examined responded less well. Control experiments showed that no mouse strain tested produced antibodies to

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                10                  20                  30
M M R K L A I L S V S S F L F V E A L F Q E Y Q C Y G S S S
          1 1 1 1 1 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0

                40                  50                  60
N T R V L N E L N Y D N A G T N L Y N E L E M N Y Y G K Q E
1 0 1 1 1 1 1 1 1 0 0 0 0 1 1 1 1 1 1 0 0 1 0 0 0 0 0 1 1 0 0

                70                  80                  90
N W Y S L K K N S R S L G E N D D G N N N N G D N G R E G K
1 1 0 0 1 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 1 0 1 1 1 1 1 1 1 0 0

               100                 110                 120
D E D K R D G N N E D N E K L R K P K H K K L K Q P G D G N
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 1 0 1 1 1 1 1 1 0 0 0

               130                 140                 150
P D P N A N P N V K P N A N P N V D P N A N P N V D P N A N
0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

     155       REPEAT      281                 290
P N A N P ..................... A N P N A N P N K N N Q G N G
0 0 0 0 0 ..................... 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0

     300                 310                 320
Q G H N M P N D P N R N V D E N A N A N N A V K N N N N E E
0 0 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 0 0 0 0 1 0 0 0 0 0 0 0 0

     330                 340                 350
P S D K H I E Q Y L K K I K N S I S T E W S P C S V T C G N
0 0 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 0 0 0 0 0 0 1 1 0 0

     360                 370                 380
G I Q V R I K P G S A N K P K D E L D Y E N D I E K K I C K
0 0 0 0 0 0 0 0 0 1 1 1 1 1 0 0 0 0 0 0 0 1 0 0 1 1 1 1 1 1 0 1

     390                 400                 410
M E K C S S V F N V V N S S I G L I M V L S F L F L N
1 1 1 1 1 1 1 1 1 1 1 1 1 1 0 1 0 0 0 0 0 0 0 0 0
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Fig. 1. The CS protein of the 7G8 strain of *P. falciparum* (*1*) as analyzed for helical amphipathic segments by AMPHI (*9*). One (1) indicates the center of an 11-residue block with periodicity of hydrophobicity like that of an amphipathic α or 3_{10} helix. Zero (0) indicates the center of blocks with other periodicities. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

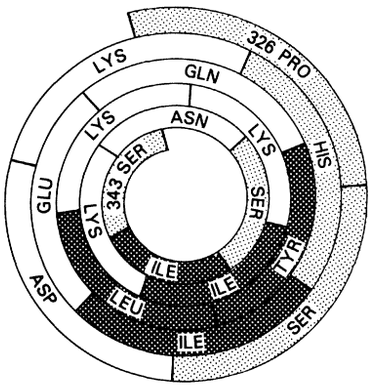

Fig. 2. Spiral diagram of CS protein 326(Pro) to 343(Ser), showing α-helical amphipathicity. View is looking down the helix from the amino terminus. Darkly shaded areas represent hydrophobic residues, stippled areas represent neutral residues, and open areas represent hydrophilic residues. The separation of hydrophobic and hydrophilic residues on opposite sides of the helix constitutes amphipathicity.

$(NANP)_n$ after inoculation with the parent vaccinia virus. As a further control, all mice responded comparably to parent vaccinia virus. These data implied that there must be one or more helper T-cell sites, apart from $(NANP)_n$, located on the CS protein, that can be recognized by B10.BR and B10.A(4R) mice.

If there were many T-cell sites located on the CS protein, many, if not most, of the different strains of mice would respond well to the CS–vaccinia virus, which expresses the entire CS protein. Because many strains do not respond, there appear to be a limited number of other major helper T-cell sites.

The fact that both the B10.BR mice, which express both I-A and I-E molecules ($I\text{-}A^k$ and $I\text{-}E^k$) and the B10.A(4R) mice which express only I-A molecules ($I\text{-}A^k$), respond suggests that this T-cell site is recognized in association with $I\text{-}A^k$. The possibility exists, however, that the B10.BR strain recognizes two T-cell sites, one in association with $I\text{-}A^k$ and one in association with $I\text{-}E^k$.

To search for candidate helper T-cell sites that might be recognized by the $I\text{-}A^k$–bearing strains of mice, we took advantage of the observation that immunodominant helper T-cell sites tend to have the ability to fold as amphipathic helices (*9, 10, 12*) and are frequently near the B-cell site (*13, 14*). Using the computer algorithm AMPHI (*9*), we analyzed the sequence of the CS protein by a least-squares fit of a sine wave to the hydrophobicity values of the amino acids along the sequence to find those segments that could best fold as amphipathic α or 3_{10} helices. The analysis (Fig. 1) indicated that a segment from residues 323 to 349 had the highest amphipathic score in the whole protein (Table 2). Because a high amphipathic score may be indicative of a T-cell site, we used this segment as a candidate for an immunodominant T-cell epitope. The am-

Table 1. Genetic control of immune response to *P. falciparum* CS protein. The results are shown as the titer of antibodies to $(NANP)_n$. Mice (five per group) were immunized intraperitoneally on day 0 and by scarification on day 21 with the CS–recombinant vaccinia (without adjuvant). Serum samples for the primary response were taken on day 20 and for the secondary response on day 35; the samples for each response were pooled prior to assay. All sera were assayed on the same day.

Strain	H-2 alleles								Titer*	
	K	A	B	J	E	C	S	D	Primary response	Secondary response
B10	b	b	b	b	(b)	b	b	b	79	111
B10.BR	k	k	k	k	k	k	k	k	79	274
B10.A(4R)	k	k	b	b	(b)	b	b	b	69	630
B10.S(9R)	s	s	s	k	k	d	d	d	<<16	60
B10.S(7R)	s	s	s	s	(s)	s	s	d	<<16	34
B10.D2	d	d	d	d	d	d	d	d	<16	16
B10.M	f	f	f	f	f	f	f	f	<16	39
B10.Q	q	q	q	q	q	q	q	q	<<16	<<16
B10.RIII	r	r	r	r	r	r	r	r	<<16	<16

* The titer is defined as the dilution of serum giving an absorbance (414 nm) of 0.5 in an ELISA assay (*19*). This definition was chosen to facilitate comparison of sera, but by a more conventional definition of titer as the highest dilution giving an absorbance significantly greater than a panel of normal mouse sera, the titers would all be numerically higher. The antigen used to coat the ELISA plates was $R32tet_{32}$.

Table 2. The predicted amphipathic segments, angles, and amphipathic scores in the CS protein of *P. falciparum*.

Midpoints of blocks	Range of angles	Amphipathic score
6–10	130–135	7.4
33–39	110–130	14.0
44–48	90–120	12.0
69–70	95–100	4.8
83–88	120–100	19.5
*108–111	135–135	7.4
*113–117	115–135	12.5
298–309	85–110	32.9
*328–344	80–100	43.5
365–369	95–135	9.2
379–383	80–100	9.8
385–397	95–130	30.7

*Regions tested in vivo.

phipathic character of this sequence is apparent when viewed as a helix in a spiral diagram (Fig. 2). The region 103 to 122 (corresponding to blocks with midpoints 108 to 117) would be amphipathic if folded as a 3_{10} helix, although the amphipathic score is lower. Because the sequence 103 to 116 had been made for other purposes, we tested it as well. Both sequences 323 to 349 and 103 to 116 have the advantage of being close to the B-cell site $(NANP)_n$. The block from 298 to 309 also meets these criteria but contains two closely spaced helix-breaking proline residues, and so was not tested in this study. The repeat region $(NANP)_n$ would not be predicted to form an amphipathic α or 3_{10} helix, although it could possibly fold as an amphipathic β structure

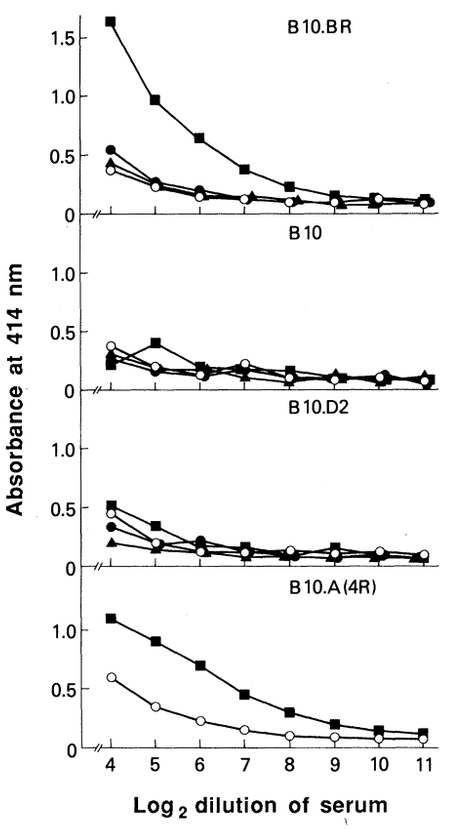

Fig. 3. B10.BR, B10, B10.D2, and B10.A(4R) mice (five per group) were immunized with peptides (100 μg) emulsified in CFA, and 6 weeks later they were challenged with the CS–vaccinia virus by scarification. Nine days after challenge, sera were collected and antibodies to $(NANP)_n$ were measured in the pooled sera from each group (*19*). The peptides used for the immunization were as follows: ■, Th2R [PSDKHIEQYLKKIKNSIS(C)], representing amino acids 326 to 343 from the sequence of the CS protein from the 7G8 strain of *P. falciparum*, plus an extra cysteine at the carboxyl terminus; ▲, EKLRK PKHKKLKQP, representing amino acids 103 to 116; ●, KPKHKKLKQPGDGNPDPN, representing amino acids 107 to 124; ○, saline. The B10.A(4R) mice were tested only with Th2R to map the genetic regulation of the positive response in B10.BR mice (see Table 1).

(periodicity of 2 or frequency 180°).

The synthetic peptide sequences [103 to 116, 107 to 124, and 326 to 343(C)] were thus made and tested for their ability to prime helper T cells (Fig. 3). Mice were primed by intraperitoneal injections with peptide emulsified in complete Freund's adjuvant (CFA) or with saline emulsified with CFA (five mice per group). Six weeks later, the mice were infected with CS–vaccinia virus by scarification in the base of the tail. Nine days later, their sera were tested. As shown in Fig. 3, B10.BR or B10.A(4R) mice primed with peptide sequence 326 to 343(C)/CFA produced significantly more antibody to $(NANP)_n$ after challenge with CS–vaccinia virus than did the control mice primed with saline/CFA. At later time points after challenge, the titers of all the antibodies increased, but the control mice developed a significant primary response to the CS–vaccinia challenge, making comparison more difficult. We refer to this T-cell site (peptide 326 to 343) as Th2R since it is the second region of the CS molecule found to contain a helper T-cell site. Other peptide sequences did not prime helper T cells in these strains. Because Th2R does not include the NANP sequence, the difference in the antibody response specific for $(NANP)_n$ between the two groups was due to T-cell priming. The CS protein encoded by the recombinant virus contains both the B-cell site $[(NANP)_n]$ and the T-cell site [Th2R] linked on the same molecule, and therefore prior immunization with Th2R can enhance specific help to the B cells that recognize $(NANP)_n$ (*15*). Mice of the B10 strain did not respond to Th2R, and so did not produce a secondary response 9 days after challenge with CS–vaccinia virus. To confirm that all of these mice contained B cells capable of recognizing $(NANP)_n$, we showed that they could produce antibodies to $(NANP)_n$ after vaccination with keyhole limpet hemocyanin conjugated to $(NANP)_n$. This is not unexpected, since the strains differ only at their H-2 loci—a region influencing T-cell recognition of antigen and indirectly the antibody responses but not affecting the immunoglobulin structural gene repertoire of B cells.

To determine if Th2R was recognized by proliferating T cells, we performed a lymph node cell transformation assay (*16*) in B10.A(4R), B10.BR, and B10 mice. The peptide was used as both immunogen and antigen. Proliferating T cells from both B10.A(4R) and B10.BR mice recognized the sequence (Fig. 4). We found consistently that the B10.A(4R) response to Th2R was greater than the B10.BR response. As a control for possible mitogenic effects of Th2R, we showed that the Th2R did not

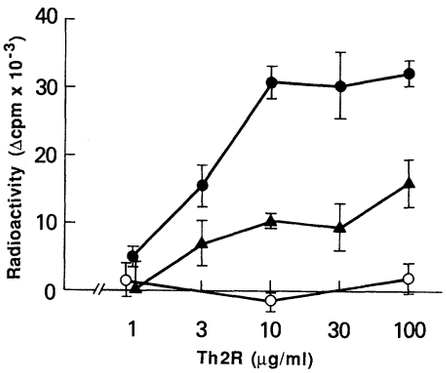

Fig. 4. B10.BR (▲), B10.A(4R)(●), and B10(○) mice (two per group) were immunized subcutaneously in the base of the tail with 100 μg of Th2R [PSDKHIEQYLKKIKNSIS(C)] emulsified in CFA. Ten days later, draining lymph node cells (4×10^5 per 0.2 ml, quadruplicate) were challenged in vitro (RPMI-1640 medium, 10% heat-inactivated fetal calf serum, $5 \times 10^{-5} M$ 2-mercaptoethanol) with different concentrations of Th2R and proliferation was assessed 4 days later by determining incorporation of [^{3}H]thymidine (New England Nuclear, 6.7 Ci/mmol) in 16 hours (1 μCi per well). Background radioactivity (no antigen in vitro) was 24,232 cpm for B10.BR, 21,387 cpm for B10.A(4R), and 16,653 cpm for B10. Vertical bars represent ±1 SE.

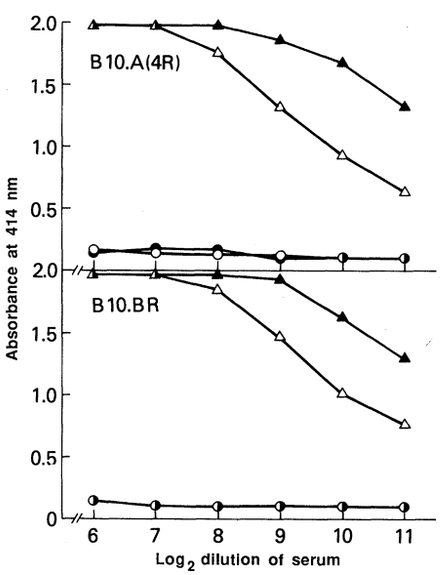

Fig. 5. B10.BR and B10.A(4R) mice (five per group) were immunized intraperitoneally with 100 μg of the conjugate Th2R-NP$(NANP)_5$NA (*18*) emulsified in CFA (△, ▲) or with 100 μg of NP$(NANP)_5$NA emulsified in CFA (○, ●) on day 0 and boosted on day 21 with aqueous antigen (30 μg). Antibody to $(NANP)_n$ was determined for pooled sera taken on day 20 (open symbols) and day 31 (closed symbols) by enzyme-linked immunosorbent assay (ELISA) (*19*).

stimulate a proliferative response in lymph node cells from immunized B10 mice. The sequence 103 to 116, while not stimulating T-cell help, could stimulate T-cell proliferation (*17*). Thus, while not all proliferating T cells are necessarily helper T cells, the proliferative response to Th2R can be correlated

with priming for help to CS–vaccinia virus.

We next constructed a synthetic immunogen containing T-cell and B-cell sites derived from the CS molecule. We prepared Th2R-NP(NANP)$_5$NA (*18*) and immunized B10.BR and B10.A(4R) mice with either this or NP(NANP)$_5$NA and then measured their antibody production in response to (NANP)$_n$. Both strains responded to the conjugate peptide but not to NP(NANP)$_5$NA alone (Fig. 5). In the native molecule, the NANP repeating unit is located between Th2R and the amino terminus, whereas in the synthetic construct, NP(NANP)$_5$NA was located at the carboxyl terminal end of Th2R. This change in orientation did not affect the ability of the T-cell site to generate carrier-specific help for the responsive B cells. Thus a synthetic immunogen was designed and constructed containing a T-cell site and a B-cell site, both of parasite origin.

While the CS protein appears to contain few major epitopes capable of stimulating T cells to help B-cell response against (NANP)$_n$, it may contain other epitopes responsible for T-cell proliferation, which may be important for antibody-independent cellular immunity. As mentioned, peptide 103 to 116 may represent such an epitope. As well, it may contain other sites recognized by cytotoxic T cells that may play a role in sporozoite immunity. While we have looked at helper T-cell epitopes that produce a B-cell response against (NANP)$_n$, other helper T-cell epitopes may be present that may preferentially help B cells of other specificities (*13, 14*); however, the specificity of the immunoglobulin antibody to (NANP)$_n$ is the one known to neutralize sporozoites. T-cell sites are required both for helper function in antibody production and for antibody-independent cellular immunity, both of which appear to be important in immunity to sporozoites (*5, 8*).

A vaccine to be used in endemic areas would rely on natural boosting from sporozoites. If natural antibody boosting by sporozoites is required, or if antibody-independent T-cell immunity is critical, a vaccine must contain parasite-derived T-cell epitopes. Natural boosting by sporozoites would maintain a high antibody titer, known to be necessary for antibody-mediated protection, as well as maintain T cells in an activated state; however, if T-cell sites were limited on the CS molecule, a vaccine reliant on natural boosting might be ineffective in some people. The more such T-cell sites that are incorporated in a vaccine, the more this problem should be minimized. The approach outlined here should be useful in the rational design of synthetic or recombinant fragment vaccines.

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18. The Th2R-NP(NANP)$_5$NA conjugate was prepared as follows: 5.7 mg of *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Pierce Chemicals) in 380 μl of dimethylformamide was added dropwise to 15 mg of NP(NANP)$_5$NA in 1.5 ml of 10 m*M* $NaPO_4$, *p*H 7.0. The mixture was stirred for 30 minutes at room temperature and then the *m*-maleimidobenzoyl peptide (MB-peptide) was separated from the unreacted MBS by desalting on a column of Sephadex G-25 in 50 m*M* PO_4, *p*H 6.0. The resulting MB-peptide pool was mixed with 15 mg of the Th2R peptide in 15 ml of phosphate-buffered saline (PBS), *p*H 7.2, and the mixture stirred at room temperature for 3 hours. The final Th2R-NP(NANP)$_5$NA conjugate was desalted on a column of Bio-Gel P-2 (Bio-Rad) and analyzed by reversed-phase high-performance liquid chromatography and its amino acid composition was determined.
19. Immulon-1 microtiter plates (Dynatek, Alexandria, VA) were coated with R32tet$_{32}$ (*3*), 100 μl per well at a concentration of 2 μg/ml. The plates were washed and the sera diluted in PBS, *p*H 7.4, containing 1% bovine serum albumin (BSA) and 0.05% Tween 20. After incubation for 1 hour at 37°C followed by washings, horseradish peroxidase–conjugated goat antiserum to mouse immunoglobulin was added. The plates were incubated again for 1 hour at 37°C, washed, and substrate added. The substrate was 2,2′-azinodi(3-ethylbenzthiazoline-sulfonate). The reaction was stopped by the addition of 1.25% KI. Optical density was read at 414 nm with a flow Titertek Multiskan.

20. We thank Smith, Kline, and French Laboratories, Swedeland, PA, for providing R32tet$_{32}$; K. Cease, W. Weiss, W. Hockmeyer, S. Ozaki, S. Brett, and M. Kojima for discussions; D. H. Sachs and T. A. Waldmann for reading of the manuscript; D. H. Sachs for providing certain mouse strains; P. Spinella and D. Pombo for laboratory assistance; and W. Davis for editorial assistance. Partial support for MFG came from a Neil Hamilton Fairley Fellowship from the National Health and Medical Research Council (Australia) and a Fulbright Postdoctoral Award.

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Apogeotropic Roots in an Amazon Rain Forest

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Roots of some tropical trees grow vertically upward on the stems of neighboring trees. Apogeotropic roots occur in 12 species across five families. These roots, originating as fine roots in the mineral soil, grow upward as fast as 5.6 centimeters in 72 hours. Apogeotropic root growth may be an adaptation to extremely low soil nutrient availability in Amazon forests. In these forests upward-growing roots obtain nutrients via the predictable pathway of precipitation that flows down along the stem. Apogeotropic roots form a nutrient cycling pathway in which nutrients are absorbed and transported directly from plant to plant, without entering the soil solution.

APOGEOTROPIC ROOTS GROW OUT of the soil and up tree stems. In a nutrient-poor Amazonian rain forest, they are an important phenomenon that constitutes an unusual and previously unreported nutrient cycling pathway. Roots of tropical trees in these forests are usually most abundant near the soil surface, although they sometimes develop into a "root mat" above the mineral soil surface (*1*). In some tropical forests, adventitious roots originating in the forest canopy absorb mineral nutrients from canopy detritus (*2*). Climbing roots, common in Amazon forests near San Carlos de Rio Negro, Venezuela (1°56′N, 67°03′W), originate in the mineral soil and the surface root mat, and sometimes grow >13 m up the outer surfaces of the bark of live trees. In a 0.1-ha area sampled in this study, all stems >4 cm dbh [diameter at breast height (137 cm)] hosted climbing roots morphologically indistinguishable from small-diameter subterranean roots. At least 12 species in Amazonia *tierra firme* (nonflooded) forest are capable of producing these apogeotropic roots. Individuals of one species, *Eperua purpurea* Bentham, send roots up their own stems; no climbing roots of other species are found on them. I propose that apogeotropic roots grow in response to a nutrient gradient caused by the flow of precipitation on the stem (stem flow) in these otherwise nutrient-scarce forests.

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