Autoantibodies to Glutamate Receptor GluR3 in Rasmussen's Encephalitis

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Rasmussen's encephalitis is a progressive childhood disease of unknown cause characterized by severe epilepsy, hemiplegia, dementia, and inflammation of the brain. During efforts to raise antibodies to recombinant glutamate receptors (GluRs), behaviors typical of seizures and histopathologic features mimicking Rasmussen's encephalitis were found in two rabbits immunized with GluR3 protein. A correlation was found between the presence of Rasmussen's encephalitis and serum antibodies to GluR3 detected by protein immunoblot analysis and by immunoreactivity to transfected cells expressing GluR3. Repeated plasma exchanges in one seriously ill child transiently reduced serum titers of GluR3 antibodies, decreased seizure frequency, and improved neurologic function. Thus, GluR3 is an autoantigen in Rasmussen's encephalitis, and an autoimmune process may underlie this disease.

Rasmussen's encephalitis is a rare, progressive, catastrophic disease of unknown pathogenesis that begins in the first decade of life, affecting previously normal children (*1*). The disease affects the cortex of a single cerebral hemisphere, resulting in intractable seizures, hemiparesis, and dementia. The diagnosis is established by these typical clinical features, together with hemispheric atrophy and a characteristic inflammatory histopathology (*1*, *2*). Treatment of the incapacitating seizures with conventional anticonvulsants is of limited benefit. Surgical removal of the affected hemisphere is the standard therapy. Glutamate and related amino acids are the predominant excitatory neurotransmitters in the mammalian central nervous system (CNS) (*3*) and have been implicated in neurodegenerative diseases and epilepsy. To generate subtype-specific antibodies to recombinant GluR proteins, we immunized rabbits with bacterially expressed *trpE* gene fusion proteins that included a portion of the putative extracellular domain of GluR1, 2, 3, 5, or 6 or the putative cytoplasmic domain of subunits (β4) of the neuronal nicotinic acetylcholine receptor (nAChR) family (*4*).

After four immunizations with GluR3 fusion protein, two rabbits developed high titers of GluR3 antibodies, anorexia, and behaviors characteristic of seizures, consisting of brief periods of immobilization, unresponsiveness, and repetitive clonic movements. One rabbit had severely lacerated its tongue, suggestive of tongue biting during a seizure. By contrast, no behavioral abnormalities were seen in another rabbit immunized with GluR3 fusion protein or in any of more than 50 rabbits injected with fusion proteins containing GluR1, 2, 5, or 6 or neuronal nAChR subunits, although high titers of the appropriate antibodies were observed (*4*). Gross examination of the brains of the symptomatic, GluR3-immunized rabbits disclosed no abnormality. However, microscopic examination disclosed inflammatory changes consisting of microglial nodules and perivascular lymphocytic infiltration mainly, but not exclusively, in the cerebral cortex, together with lymphocytic infiltration of the meninges (Fig. 1). Microscopic examination of the brain of the asymptomatic rabbit immunized with GluR3 fusion protein disclosed no abnormality. We reasoned that the rabbits' symptoms and inflammatory CNS histopathology probably represented an autoimmune process directed against GluR3, on the basis of the immunization history, the occurrence of symptoms only in GluR3-immunized rabbits, and the similar distribution of inflammatory pathology in these animals and GluR3 mRNA in normal animals (*5*). The occurrence of disease in some, but not all, GluR3-immunized rabbits is similar to the incidence of autoimmune myasthenia gravis in mice immunized with nAChR (*6*).

These symptomatic rabbits resembled individuals with Rasmussen's encephalitis. Common features include (i) recurrent seizures (*1*); (ii) inflammatory histopathology, characterized by meningeal and perivascular lymphocytic infiltrates and microglial nodules (Fig. 1) (*2*); and (iii) predominant

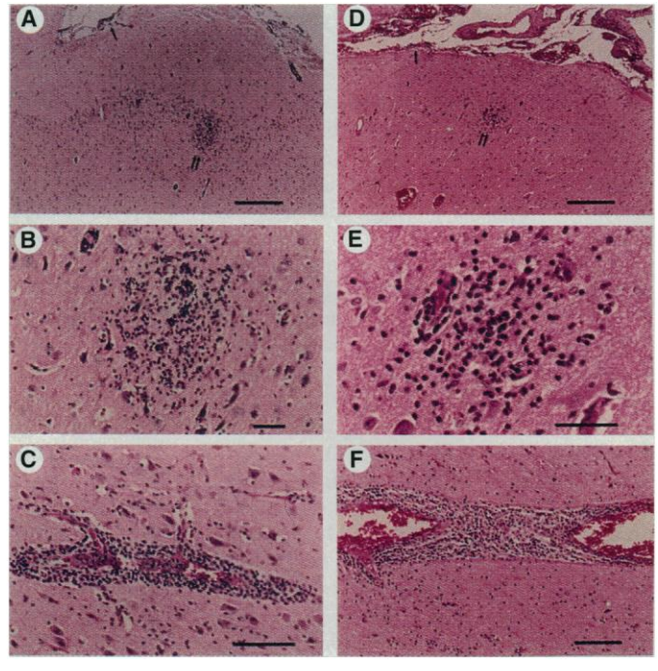

Fig. 1. Characteristic histopathology of neocortex of one symptomatic rabbit immunized with GluR3 (**A** through **C**) and comparable sections from resected temporal lobe of individual AI (**D** through **F**). (A) and (D) Neocortex with meningeal lymphocytic infiltrate (arrow) and a microglial nodule (double arrow). Bars are 200 μm. (B) and (E) Microglial nodules from (A) and (D). Bars are 50 μm. (C) and (F) Perivascular lymphocytic cuffing from entorhinal cortex in the rabbit and amygdala in individual AI. Bars are 100 μm.

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localization of this inflammatory process in the cerebral cortex with relative sparing of the basal ganglia, thalamus, deep white matter, brainstem, and cerebellum (*2*). We therefore hypothesized that Rasmussen's encephalitis is due to an autoimmune process directed at GluR3 protein.

To test this idea, we measured immunoreactivity toward GluR3 and other neural receptors in sera from four individuals with pathologically confirmed Rasmussen's encephalitis, four age- and sex-matched epileptic children, four age- and sex-matched children without CNS disease, five children with active CNS inflammation, four other epileptic children, and four other normal children. Protein immunoblot analysis was performed with *trpE* fusion proteins containing corresponding regions of the putative extracellular domains of GluR1, 2, 3, and 5 (Fig. 2 and Table 1) and a portion of the putative intracellular domain of nAChR subunit β4 (*4*, *7*, *8*); blots were interpreted by observers unaware of the sources of the sera. Immunoreactivity to GluR3 fusion protein was detected in multiple sera samples from two individuals with Rasmussen's encephalitis [CK in (*9*); AI in Fig. 2]. CK also exhibited weak immunoreactivity to GluR2 fusion protein. Serum from a third individual with Rasmussen's encephalitis (EM) exhibited weak immunoreactivity to GluR2 fusion protein but not to other antigens (*9*). The fourth individual with Rasmussen's encephalitis (GO) did not exhibit serum immunoreactivity to any tested antigen. Sera from 20 of 21 control individuals showed no immunoreactivity, and one control serum showed immunoreactivity to GluR3 (*10*) that was different from the serum GluR immunoreactivity exhibited by individuals with Rasmussen's encephalitis ($P = 0.006$; Fisher's exact test).

To obtain independent validation of GluR immunoreactivity in these sera, we examined immunoreactivity toward full-length GluR3 protein in its three-dimensional conformation. Human embryonic

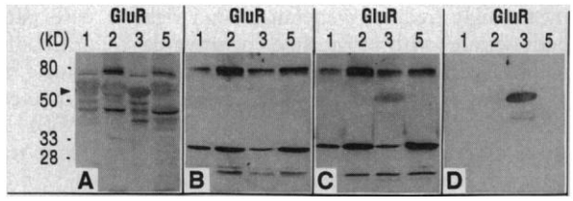

Fig. 2. Prominent serum reactivity toward GluR3-*trpE* fusion protein in an individual with active Rasmussen's encephalitis. Protein extracts enriched in fusion proteins were loaded on the designated lanes (GluR1, 2, 3, and 5) and visualized (*4*, *8*). The site at which GluR fusion proteins reside is denoted by the arrowhead in (A). (**A**) Serum immunoreactivity of a rabbit immunized with *trpE* protein devoid of GluR protein. Because each GluR fusion protein contains *trpE* protein, each GluR fusion protein exhibits immunoreactivity. The bands not marked by the arrowhead represent irrelevant immunoreactivity, principally to bacterial proteins or to fragments of GluR fusion protein. (**B**) No immunoreactivity to any GluR fusion protein is evident in serum from the individual (GO) without active disease. This blot was typical for sera of 20 of 21 controls. (**C**) Serum from individual AI shows immunoreactivity to GluR3, but not to any other GluR. (**D**) We first adsorbed serum from individual AI with lysate from *trpE*-expressing *E. coli* to remove immunoreactivity to bacterial antigens (*4*, *8*). The main GluR3 immunoreactivity is shown, with smaller faint bands presumably representing GluR3 fusion protein degradation products. Of the individuals tested, this serum exhibited the most prominent immunoreactivity to GluR3. Molecular size standards are shown at left (in kilodaltons).

Table 1. Summary of serum immunoreactivity to GluR in individuals with Rasmussen's encephalitis. Initials of all individuals were changed to protect their identity. Sz, seizures; GM Sz, generalized, tonic clonic seizures; R1, GluR1; and R6, GluR6 transiently expressed in transfected cells.

Diagnoses	Immunoreactivity	
	Immunoblot	In cells
	Rasmussen patients	
AI, progressive	GluR3	GluR3 (not R1 or R6)
CK, progressive	GluR3; weak GluR2	GluR3 (not R1 or R6)
EM, hemiparesis, Sz	Weak GluR2	GluR3 (not R1 or R6)
GO, stable, no Sz	None	None
	Age- and sex-matched epileptic controls	
Absence + GM Sz	None	None
Simple, partial Sz	None	None
Complex, partial Sz	None	None
Posttraumatic Sz	None	None
	Children with active CNS inflammation	
CNS lupus erythematosus	None	None
CNS lupus erythematosus	None	None
Multiple sclerosis	None	None
Varicella encephalitis	None	None
Tuberculous meningitis	None	None
	Age- and sex-matched normal controls	
Cardiac surgery	GluR3	None
Trauma	None	None
Trauma	None	None
Obesity	None	None
	Epileptic children	
Four children	None	None
	Normal children	
Four children	None	None

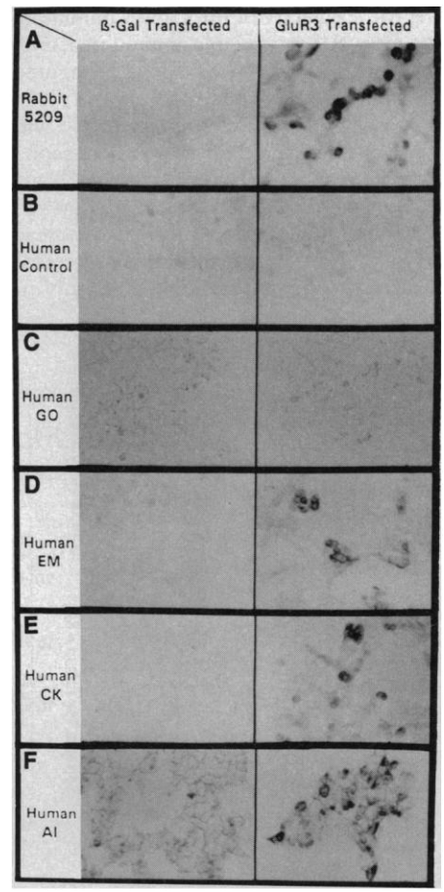

Fig. 3. Serum immunoreactivity toward transfected HEK 293 cells expressing either GluR3 or β-galactosidase (*11*). (**A**) Serum from one symptomatic, GluR3-immunized rabbit reacts with cells expressing GluR3. (**B**) Sera from control individuals exhibit no immunoreactivity. Immunoreactivity to transfected cells expressing GluR3 is present in sera of those individuals with active Rasmussen's encephalitis: EM (**D**), CK (**E**), and AI (**F**) but not in individual GO (**C**), who exhibits no active disease.

kidney (HEK) 293 cells were transiently transfected with expression plasmids containing the complementary DNA (cDNA) for GluR3 or β-galactosidase (*11*). Sera from the three individuals with Rasmussen's encephalitis that exhibited immunoreactivity to GluR3 or GluR2 on protein immunoblots also reacted with transfected cells expressing GluR3 (Fig. 3 and Table 1). Serum from the fourth individual with Rasmussen's encephalitis, which was negative by protein immunoblot analysis, did not react with transfected cells. None of the control sera reacted with transfected cells, including the single control serum that was positive on the protein immunoblot (*10*). The correlation between serum immunoreactivity to GluR3 and Rasmussen's encephalitis was significant ($P = 0.002$; Fisher's exact test). Sera from individuals with Rasmussen's encephalitis did not react with transfected cells expressing the closely related GluRs GluR1 or GluR6, demonstrating the specificity of the GluR3 immunoreactivity. All measurable immunoreactivity in these individuals was of the immunoglobulin G (IgG) class.

GluR immunoreactivity correlated with clinical findings of Rasmussen's encephalitis. The three individuals (AI, CK, and EM) with GluR immunoreactivity on protein immunoblot and GluR3 immunoreactivity on transfected cells have progressive disease or ongoing seizures. The only individual (GO) with Rasmussen's encephalitis without immunoreactivity to any GluR proteins by protein immunoblot or transfected cell analyses underwent hemispherectomy 2 years before data collection and has since remained clinically stable and seizure-free.

The correlation between GluR3 immunoreactivity and disease activity suggested that GluR3 antibodies could be pathogenic in Rasmussen's encephalitis. We therefore hypothesized that removal of GluR3 antibodies by recurrent plasma exchange (PEX) would be beneficial. CK, now 9 years old, was well until a minor, left forehead injury in 1990, following which she experienced increasingly frequent generalized and right body seizures with progressive cognitive decline, speech disability, right hemiparesis, and left cerebral atrophy. No remissions occurred. Pathologic examination of a left temporal cortex biopsy confirmed the diagnosis of Rasmussen's encephalitis. GluR3 immunoreactivity, monitored with an enzyme-linked immunosorbent assay (ELISA) (*4, 12*), was high. She was treated with recurrent, single-volume PEX and exhibited a beneficial response (Fig. 4). During the first 7 weeks of PEX, seizure frequency decreased by 80%. Cognition, speech, and hemiparesis improved, correlating in time with diminished GluR3 immunoreactivity. Over the ensuing 4 weeks, however, seizure frequency increased, and cognition, speech, and motor skills deteriorated in parallel with increased GluR3 immunoreactivity.

Molecular mimicry between self and foreign antigens expressed in microbes is one mechanism that has been proposed for the pathogenesis of autoimmune diseases (*13*). Structural similarities have been identified between the ligand-binding domains of multiple classes of GluRs and bacterial periplasmic amino acid–binding proteins (*14*). Infection with such microbes may induce an immune response that also targets GluRs.

The transient improvement after PEX in one seriously ill child suggests that circulating antibodies contribute to disease pathogenesis. The circulating autoantibodies would have to gain access to GluR3 in the brain, which is normally protected by the blood-brain-barrier (BBB). Focal disruption of the BBB can occur transiently with focal seizures (*15*) or as a consequence of head injury (*16*), an event that occasionally precedes Rasmussen's encephalitis (*2, 17*). After disruption of the BBB, we hypothesize that the ensuing immune-mediated neural injury could trigger focal seizures. Thus, a cycle could be set in motion whereby focal seizures disrupt the BBB and facilitate the local access of pathogenic antibodies to brain antigens; the subsequent immune response could cause more seizures; and a progressive disorder results. This cycle could also explain the localized cortical onset of disease and the gradual expansion throughout one hemisphere. The contralateral hemisphere would be spared because it would not be a source of focal seizures, and the BBB would be relatively preserved.

Our data establish a link between circulating antibodies to a ligand-gated ion channel receptor of the CNS and a progressive encephalopathy with epileptic seizures. A related process may be operative in other forms of epilepsy, because histopathology similar to that of Rasmussen's encephalitis has been identified in 5 to 10% of individuals undergoing temporal lobectomy for refractory epilepsy (*18*). Related processes may also contribute to other CNS disorders with inflammatory histopathology.

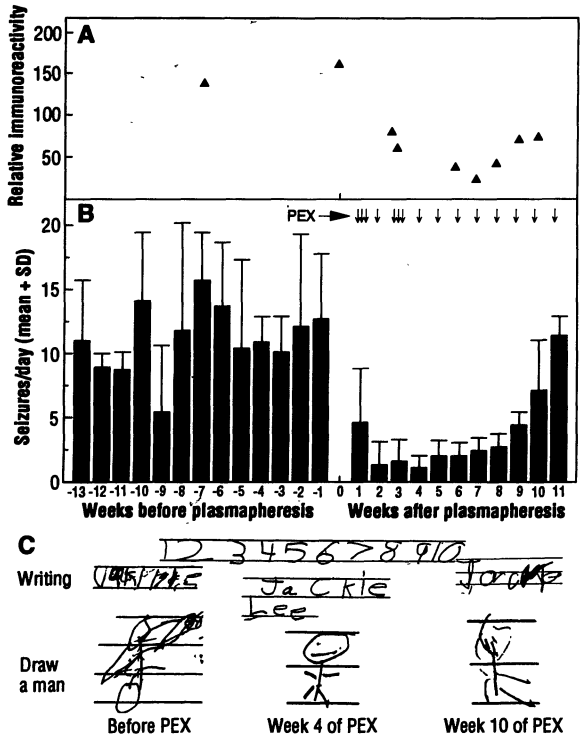

Fig. 4. Correlation of seizure frequency (**B**), neurologic function (**C**), and GluR3 immunoreactivity (**A**) in individual CK before and during 11 weeks of repeated PEX [indicated by arrows in (B)]. Before PEX, GluR3 antibody titer was high. She had 10 to 12 right body or generalized seizures daily, mild right hemiparesis, and no spontaneous speech. She was unable to read or solve simple math problems, and her writing and drawing were primitive. During the first 7 weeks of PEX, GluR3 antibody titer measured immediately before each PEX decreased, in temporal association with reduced seizure frequency. She spoke spontaneously in phrases and short sentences; her understanding, right hemiparesis, calculations, drawing, and writing improved. For the first time in 2 years, she resumed playing with dolls, riding a bicycle, and participating in household activities. During the last 4 weeks of PEX, GluR3 antibody titers gradually increased, seizure frequency increased, and cognitive skills declined. Numbers in (C) were written by the individual during week 4 of PEX. We omitted the individual's last name from her writing during week 4 of PEX to protect her identity. During PEX, other therapies were unchanged.

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8. Sera were numbered and frozen. GluR and β4-nAChR *trpE* fusion protein constructs were produced and analyzed as described (*4, 7*), except we used 2% dry milk (Blotto) to block blots. Blots were incubated overnight at 4°C in primary serum diluted 1:1000 in Blotto, then in goat antibody to human IgG conjugated to alkaline phosphatase diluted at 1:750 in Blotto for 1 hour at 25°C, and then developed (*4*). We removed *Escherichia coli* background by incubating a suspension of lysed, *trpE*-expressing bacteria in the serum sample for 2 hours at 25°C and then centrifuging it. In another examination, each protein immunoblot was photographed, coded, and interpreted blindly by three investigators; interpretations were congruent in 73 of 76 samples (*9*).
9. S. W. Rogers *et al.*, unpublished data.
10. The single control individual whose serum exhibited immunoreactivity to GluR3 fusion protein by protein immunoblot analysis was sampled 1 week after open heart surgery. Cardiac bypass under hypothermic anesthesia may be a nonspecific activator of the immune system [J. R. Utley, *J. Cardiac Surg.* **5**, 177 (1990)]. Her serum was not immunoreactive with GluR3 expressed in transfected cells, suggesting that the immunoreactivity detected by protein immunoblot analysis may not be to native GluR3.
11. HEK 293 cells were transfected with expression plasmids containing the cDNA for either GluR1, 3, or 6 or the bacterial protein β-galactosidase (*4, 7*). The serum from individual AT exhibited nuclear immunoreactivity that we removed by adsorbing the serum samples with HEK 293 cells transfected with the parent expression plasmid without an insert (*4*). Sera were coded and tested in two laboratories independently (T.E.H. and S.W.R.). Sera from immunized rabbits served as the positive controls.
12. ELISA assays used either GluR3 or β4-nAChR fusion proteins that were solubilized and adsorbed to microtiter plates (Immulon) (*4, 7*). Wells were blocked with Blotto for 2 hours at 25°C before we added sera at various dilutions for 1 hour at 25°C. We observed immunoreactivity using goat antibody to human IgG, conjugated with peroxidase, and 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (1 mg/ml) in McIlvain's buffer (pH 4.6) and 0.0005% hydrogen peroxide. Duplicate samples were scanned at 405 nM with an ELISA reader (Teritek). We determined GluR3-specific immunoreactivity by subtracting β4-nAChR reactivity from GluR3 reactivity.
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19. We thank D. V. Lewis and G. R. DeLong for access to their patients and I. Verma for use of his cell culture laboratory. Supported by the National Institute of Neurological Disease and Stroke NS30990R29 (to S.W.R.), NS28709 (to S.F.H.), NS17771 and NS24448 (to J.O.M.), Research to Prevent Blindness and the National Eye Institute EY08362 (to T.E.H.), and the NIH General Clinical Research Centers Program to Duke University (MO1-RR-30).

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Variable Gearing During Locomotion in the Human Musculoskeletal System

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Human feet and toes provide a mechanism for changing the gear ratio of the ankle extensor muscles during a running step. A variable gear ratio could enhance muscle performance during constant-speed running by applying a more effective prestretch during landing, while maintaining the muscles near the high-efficiency or high-power portion of the force-velocity curve during takeoff. Furthermore, during acceleration, variable gearing may allow muscle contractile properties to remain optimized despite rapid changes in running speed. Force-plate and kinematic analyses of running steps show low gear ratios at touchdown that increase throughout the contact phase.

Toes were present in the earliest tetrapods (*1*) and occur in all modern tetrapods except those that are highly specialized for limbless or aquatic locomotion. Feet and toes form an adaptable interface between the animal and its environment. They provide traction and a means for grasping the substrate, function as various tools and weapons (*2*), and help to maintain balance (*3*). In this report, we suggest that feet and toes improve locomotor performance by varying the gear ratio (that is, the velocity ratio) between the ankle extensor muscles and the point of application of the force on the ground (the center of force) during the course of the contact phase of a running step.

The proposed mechanism is easily visualized during running at a steady speed (Fig. 1). The foot is analyzed as a simple Type 1 lever of zero mass with the fulcrum at the ankle by means of the equation $R \times F_r = r \times F_m$, where R is the ground force moment arm, F_r is the ground reaction force, r is the muscle force moment arm, and F_m is the muscle force. During the contact period of a step, the point at which force is applied to the ground (the center of force) moves from under the heel or middle portion of the foot at touchdown to the tips of the toes at takeoff. This forward translation could increase the length of the moment arm between the ankle and the force exerted on the ground (R) and, therefore, increase the gear ratio (R/r) of the ankle extensor muscles and tendons.

Variable gearing would be advantageous in running, as it is in the automobile, because in both cases the motors (cross-bridges in muscle and pistons in engines) have a limited speed range over which they operate at peak power or efficiency (*4*). In order to maintain a narrow range of optimal engine speeds despite varying drive speeds, the ratio of engine speed to drive speed must be changed by a variable gear ratio. Furthermore, muscles have unique properties that can benefit from variable gearing within the contact phase of a running step. Active muscles that are forcibly stretched just before shortening are able to do more work during the shortening. This nonelastic enhancement of the contractile properties of the muscle increases, within limits, with increasing stretch length (*5*) but is effective over relatively small shortening distances. If a runner were to land at a low gear ratio and take off at a higher gear ratio, both the prestretch and the subsequent shortening of the muscles could be optimized. Thus, variable gearing could reduce the need for locomotor specialization, allowing individuals to move about more efficiently, accelerate more quickly, run faster, and jump higher.

To test this hypothesis, five people (3 males and 2 females) ran over a Kistler 9281B force plate. Four of these people also accelerated maximally over the force plate, starting just off the plate so that the first step landed on the plate. A lateral view of limb position was recorded with a Peak high-speed video camera at 120 images per second (Fig. 1). Recordings of forces on the ground allowed calculation of the magnitude and orientation of the ground reaction force and the position of the center of force under the foot. For each video image taken during foot support (at 8.33-ms intervals), the ground force moment arm (R) was calculated by dividing the moment at the ankle by the resultant of the horizontal and vertical ground forces. Similarly, the mus-

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