also known that pretreatment of PHA-stimulated PBMC cultures with the protein synthesis inhibitor cycloheximide abolishes the PHA-induced accumulation of c-*myb* mRNA but not c-*myc* mRNA (*24*). We have also observed that c-*myc* mRNA is induced in c-*myb* antisense–treated PBMC after PHA addition (*25*). These results indicate that T lymphocyte proliferation requires the function of at least two nuclear proto-oncogenes and suggest that c-*myb* regulates T lymphocyte proliferation subsequent to the activation of c-*myc* expression. Finally, these studies suggest that c-*myb* antisense oligomers may eventually prove useful as immunosuppressive agents.

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Mechanics of Stimulated Neutrophils: Cell Stiffening Induces Retention in Capillaries

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The effect of peptide chemoattractants on neutrophil mechanical properties was studied to test the hypothesis that stimulated neutrophils (diameter, 8 micrometers) are retained in pulmonary capillaries (5.5 micrometers) as a result of a decreased ability of the cell to deform within the capillary in response to the hydrodynamic forces of the bloodstream. Increased neutrophil stiffness, actin assembly, and retention in both 5-micrometer pores and the pulmonary vasculature were seen in response to *N*-formyl-methionyl-leucyl-phenylalanine. These changes were abolished in cells that had been incubated with 2 micromolar cytochalasin D, an agent that disrupts cellular actin organization. A monoclonal antibody directed at the CD11-CD18 adhesive glycoprotein complex did not inhibit the increase in stiffness or retention in pores. These data suggest that neutrophil stiffening may be both necessary and sufficient for the retention that is observed. Hence, neutrophil sequestration in lung and other capillaries in the acute inflammatory process may be the result of increased stiffness stimulated by chemoattractants.

CIRCULATING NEUTROPHILS ARE RETAINED within the lung microvasculature after exposure to systemic or local intrapulmonary stimuli (*1*). Under these circumstances, neutrophils are retained almost exclusively within pulmonary capillaries, whereas they are localized in postcapillary venules during inflammation in tissues supplied by the systemic circulation (*2*). The mechanisms by which neutrophils are localized at this site are unknown, but neutrophil-neutrophil adherence (leukoaggregation) (*3*) and neutrophil-endothelial adherence (*4*) have been suggested. Vedder and colleagues have shown, however, that a monoclonal antibody directed against the CD11-CD18 adhesion-related glycoproteins inhibited the shock-induced retention of neutrophils in the gut microvasculature, but had no effect on retention of neutrophils in the lung (*5*), suggesting that adhesion per se may not be responsible. Because neutrophils [mean diameter, 8 μm (*6*)] are larger than the pulmonary capillary [mean diameter, 5.5 μm (*7*)], we examined the possibility that stimulated retention of neutrophils results from a diminished ability to deform during capillary transit.

Earlier studies showed that neutrophils are deformed by passage through 5-μm diameter pores in vitro and that retention in this system is sensitive to the hydrodynamic properties of the flow stream, as is retention in the pulmonary microcirculation (*8*). Furthermore, the response of cells in pores to hydrodynamic stress is different from that of cells adherent to a surface (*9*), suggesting that geometric constraints similar to those encountered during capillary transit drive neutrophil deformation. A prominent feature of the neutrophil response to stimulation by *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) is cytoskeletal (particularly microfilament) assembly (*10*), and studies of nonmyeloid cells show a relation between changes in cytoskeletal organization and alterations in the mechanical properties of the cell (*11*). Accordingly, we hypothesized that organization of the actin-containing cytoskeleton accompanying stimulation made the neutrophil rigid enough to be retained within capillaries.

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Direct measurements of cell stiffness are needed to distinguish the roles of deformation and adhesion in the retention of neutrophils in capillaries. Micropipette aspiration measurements have characterized the viscoelastic behavior of neutrophils in the resting state (*12*). After stimulation, neutrophils with both lower and higher stiffness values than the control cells have been observed (*13*), and pseudopods, which contain an extensive array of microfilaments, are stiffer than the rest of the cell (*14*). We have used the "cell poker" (*15*), which measures the force required to indent the free surface of an adherent cell, to assess the viscoelastic properties of neutrophils under resting and stimulated conditions. This provides a characterization of cellular deformability, the stiffness, which is the force resisting indentation (in millidynes) per unit of indentation (in micrometers). Unstimulated human neutrophils showed a stiffness of 0.054 ± 0.036 mdyne/μm (median ± SD; $n = 63$), as shown in a representative tracing from an unstimulated cell (Fig. 1A). This value is slightly less than stiffness values for murine lymphocytes and rat basophilic leukemia cells (*16*) and considerably less than those reported for firmly adherent cells such as fibroblasts (*15*). However, after stimulation with the chemoattractant FMLP, neutrophils rapidly stiffened as assessed by the increase in the slope of the force-displacement curve. A representative tracing from a cell after stimulation with $10^{-8}M$ FMLP is shown (Fig. 1B). Moreover, after stimulation the hysteresis in the force-displacement curves increased [as assessed by different characteristics of the out-going limb (Fig. 1B)], suggesting an increase in viscous forces within the cell. Similar increases in the stiffness of lymphocytes and rat basophilic leukemia cells responding to receptor aggregation have been reported (*16*).

A wide range of stiffness was observed within the stimulated neutrophil populations (*17*) (Fig. 1C). Measurements on unstimulated cells clustered closely around the median stiffness. Exposure to $10^{-10}M$ FMLP increased the stiffness of a small fraction of the population, whereas exposure to $10^{-9}M$ and $10^{-8}M$ FMLP increased the proportion of responding cells and the median stiffness. Finally, $10^{-6}M$ FMLP increased median stiffness still further with no apparent plateau (*18*).

The effect of these structural alterations was assessed by measuring retention of neutrophils within 5-μm pores perfused at 5 ml/min. Retention was increased when the cells were exposed to FMLP, although the effective pressure gradient (*8*) and hence the shear stress of 200 dynes/cm^2 was unchanged, an indication that the properties of the neutrophil itself had changed. Since mechanical properties of some cells appear to reflect the degree and organization of actin assembly (*11*), actin polymerization in stimulated neutrophils was measured with *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phallicidin (NBD-phallicidin) (*10*) at 30 s after exposure to FMLP. The concentration dependence of neutrophil retention in pores was nearly identical to that of FMLP-induced actin assembly (*10, 19*) as there was a small but significant effect at $10^{-10}M$ FMLP and an apparent plateau at $10^{-8}M$ FMLP.

Since FMLP increases neutrophil adherence as well as stiffness (*20*) it was critical to determine the relative roles of each in inducing retention in pores. Stimulation of adherence requires, in general, higher concentrations of FMLP than do increased retention, actin assembly, or stiffness. Nevertheless, we tested more directly the relative importance of stiffness and adherence by measuring the effects of disrupting actin filament organization with cytochalasin D (CD) (*21*) and of inhibiting adherence with the monoclonal antibody 60.3 (MAb 60.3) (*22*). Cytochalasin D (2 μ*M*) completely prevented the increase in stiffness after exposure of cells to $10^{-8}M$ FMLP (*23*) but had no effect on adherence, whereas MAb 60.3 (44 μg/ml) inhibited adherence but had no effect on stiffness (Fig. 2, A and B). Likewise, retention in pores was inhibited by CD, but not by MAb 60.3 (Fig. 2C).

To determine whether retention of neutrophils in vivo might occur by similar mechanisms, we measured the effect of CD on the retention in the lung of neutrophils treated with FMLP ex vivo. Rabbit neutrophils, isolated and labeled with ^{111}In (*24*), were incubated with buffer or CD (2 μ*M*) for 5 min, then incubated with $10^{-8}M$ FMLP for 30 s before being infused into recipient rabbits. We quantified the interaction of the infused neutrophils with the lungs of the recipient animals by constructing regions of interest around the image of the lungs after scanning with a scintigraphic camera (*24*). Control cells not treated with CD or FMLP passed fairly rapidly through the lungs, whereas neutrophils exposed to FMLP were retained (*25*). The increased retention induced by FMLP was prevented by CD (Fig. 3).

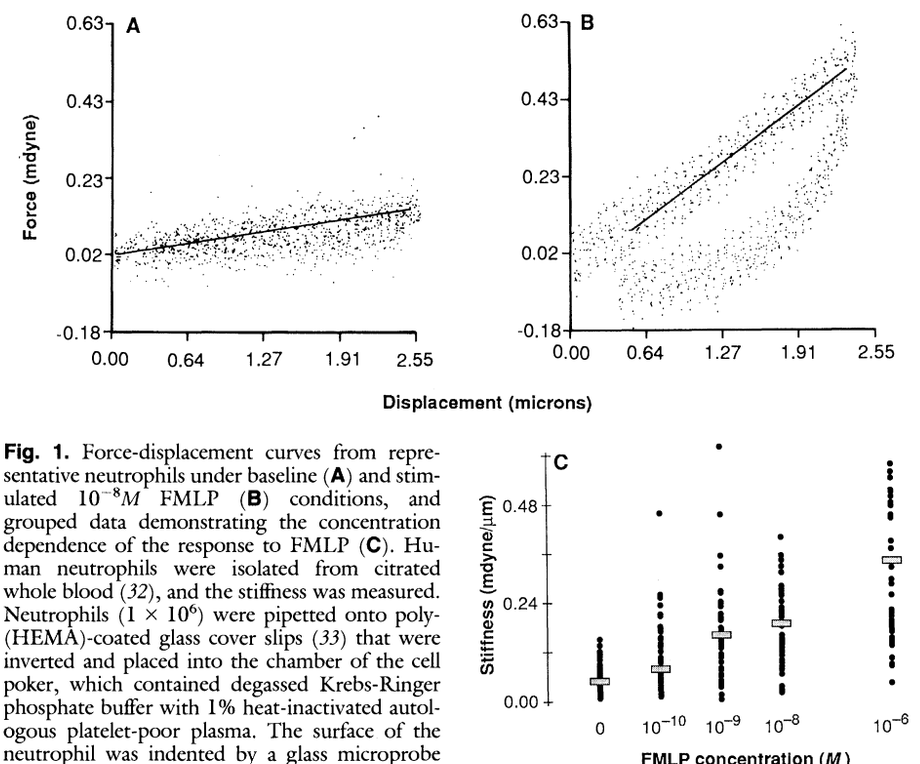

Fig. 1. Force-displacement curves from representative neutrophils under baseline (**A**) and stimulated $10^{-8}M$ FMLP (**B**) conditions, and grouped data demonstrating the concentration dependence of the response to FMLP (**C**). Human neutrophils were isolated from citrated whole blood (*32*), and the stiffness was measured. Neutrophils (1×10^6) were pipetted onto poly-(HEMA)-coated glass cover slips (*33*) that were inverted and placed into the chamber of the cell poker, which contained degassed Krebs-Ringer phosphate buffer with 1% heat-inactivated autologous platelet-poor plasma. The surface of the neutrophil was indented by a glass microprobe (tip diametrer ~2 μm) attached to a flexible glass beam of known bending constant. Neutrophils were indented at their approximate center. Maximum depth of indentation was <2.6 μm and the velocity of indentation was 5.1 μm/s. The degree of bending of the beam was used to calculate the stiffness of the cell, defined as the slope of the in-going limb of the force-displacement curve [(A) and (B)]. Data from the out-going limb were not fitted, but demonstrate hysteresis (B). The stiffness values for the representative cells in (A) and (B) are 0.05 and 0.23 mdyne/μm. Each point in (C) represents a single cell from one of five donors. For each donor, between 30 and 82 cells were studied. The stiffening response to FMLP (C) is concentration-dependent and significant ($P < 0.05$, Wilcoxon rank sum). Although cells from each donor were not studied at each FMLP concentration, there was no significant difference between donors.

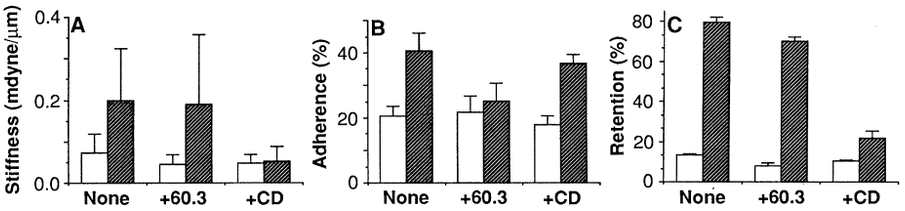

Fig. 2. Effects of CD and MAb 60.3 on (**A**) stiffness, (**B**) adherence, and (**C**) retention in pores. Neutrophil adherence to a protein-coated plastic surface was measured as described (*20*), and the bars represent mean ± SE based on three determinations, each done in quadruplicate. The effects of CD and MAb 60.3 were assessed by incubating these agents at the indicated concentration with the cells for 5 min at 37°C before addition of FMLP; the inhibitors were included in the assay. Stiffness was measured as described above, and the bars represent median ± SD of 51 cells for CD, and 25 cells for MAb 60.3. Retention of neutrophils in filters was performed as previously described (*8*), and bars represent mean ± SE of four observations, each done in triplicate. Hatched bars, FMLP; blank bars, buffer.

Inflammation in systemic vascular beds has been ascribed to neutrophil accumulation in the postcapillary venule (*2*). Neutrophil behavior in both pulmonary and systemic capillaries, however, may be pathophysiologically important. Under normal conditions, cells are retained in the low-pressure pulmonary capillaries to form the marginating pool (*26*) and, to a smaller exent, in the high-pressure systemic capillaries (*27*) in a way that may be related to the local shear stress (*9*). When the systemic pressure is lowered, however, more neutrophils are retained longer in systemic capillaries and contribute to the no-reflow phenomenon and the initiation of ischemic injury (*28*). We suggest that the difference in neutrophil retention between the pulmonary and systemic circulations is partly due to the perfusion pressure and hence to the force (shear stress) driving neutrophil deformation. Furthermore, neutrophils exposed to stimuli are retained in, and migrate across, pulmonary capillaries (*1*). The size differences between capillaries and neutrophils requires that one or both must deform during transit. Hence control of deformation could be a regulator of microcirculatory flow. The importance of cell stiffness in retention is further exemplified in the hyperleukocytic leukemias, wherein leukostatic plugging of the lungs and other organs is more common with stiff myelocytic cells than with the more deformable lymphocytic leukemia cells (*29*). Although we have focused here on neutrophil deformability, the deformability of the capillary may also affect neutrophil transit.

The stiffened neutrophil is retained within capillary-sized pores in vitro and the pulmonary microcirculation in vivo, but adhesion of neutrophils to the endothelial surface could also enhance retention. Although MAb 60.3 did not prevent neutrophil retention within pores, the relation between expression of the CD11-CD18 complex and adherence is not precise (*30*) and other adhesive interactions may be important. The role of the stiffness response suggests that cell surface interactions that require very close apposition [analogous to coulomb friction (*31*)] may become important during deformation in capillaries. These forces might thus not be evident in venules or if the neutrophil were prevented from becoming stiff.

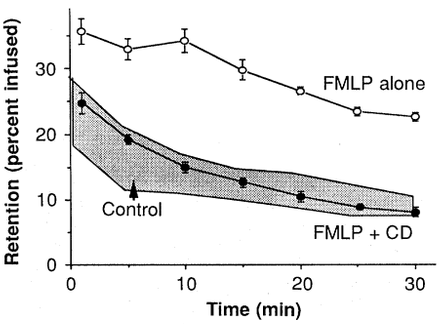

Fig. 3. Effect of CD on retention of rabbit neutrophils in the lung. Neutrophils labeled with ^{111}In were washed, resuspended in plasma, and incubated with buffer (n = 4) or CD (n = 4) for 5 min at 37°C before addition of $10^{-8}M$ FMLP. Immediately on exposure of the cells to FMLP, neutrophils (50×10^6 per rabbit) were infused into the marginal ear vein of recipient rabbits (*34*) and neutrophil transit through the lung was assessed by external gamma scintigraphy (*24*) and is expressed as a fraction of total radioactivity infused. The stippled portion represents 2 SD on either side of a line determined from five normal rabbits receiving untreated donor neutrophils. Error bars represent ± SE.

Reorganization of the neutrophil cytoskeleton in response to chemoattractants, and the attendant increase in cytoplasmic stiffness, may be critical for neutrophil retention in pulmonary capillaries and the initiation of pulmonary inflammation. In addition, when ischemic events occur and systemic circulation flow and pressure decrease, capillary retention of neutrophils in systemic microvascular beds due to increased stiffness may contribute to the pathogenesis of a wide range of disorders, including sepsis, myocardial infarction, and post-traumatic shock.

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17. All data are reported as mean ± SE unless otherwise specified. Analysis of variance (ANOVA) was used for the comparison of means with corrections for multiple comparisons (Ryan-Einot-Gabriel-Welsh) where appropriate. For the analysis of the data from the cell poker experiments, a nonparametric test (Wilcoxon rank sum) was used because the data were not normally distributed, and the data are reported as median ± SD.
18. These data also vary as a function of time after stimulation, such that pooled data for a given treatment include cells in different phases of the time response. To analyze the time response, we obtained measurements of stiffness and actin assembly at different times after stimulation, which were then fitted to an exponential time decay by nonlinear regression analysis. The curves describing the response to $10^{-9}M$ and $10^{-8}M$ FMLP are fitted by an exponential decay with τ = 368 and 1608 s, respectively, whereas the response to $10^{-6}M$ FMLP was independent of time over the measuring period. Hence the time constant was long compared to the measuring period with $10^{-6}M$ FMLP and progressively shorter with $10^{-8}M$ and $10^{-9}M$ FMLP. Thus the observed increase in median stiffness occurs from both an increase in maximal cell stiffness and an increase in the duration of the response with increased FMLP concentration.
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23. The lack of effect of CD on the deformability of resting cells could indicate that other structures, such as other filament systems or the nucleus, are dominant in this state. The stiffness of resting neutrophils is close to the lower limit of detection of the cell poker, and it is possible that a small decrease in stiffness might not be detected.
24. Rabbit neutrophils [C. Haslett *et al.*, *Am. Rev. Resp. Dis.* **136**, 9 (1987); G. P. Downey *et al. J. Appl. Physiol.* **64**, 728 (1988)], like human neutrophils,

were stimulated to adhere and assemble actin by FMLP. Furthermore, CD did not inhibit adherence, but did disrupt the microfilament organization in a fashion similar to that seen with human neutrophils.
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33. Adherence of the cell to a rigid substratum promotes spreading, which may increase the measured stiffness. Both spreading and the consequent increase in stiffness may be prevented by plating the cells on polyhydroxyethyl methacrylate [poly(HEMA)] [J. Folkman and A. Moscona, *Nature* **273**, 345 (1978); B. B. Daily, thesis, Washington University, St. Louis, MO (1985)]. Similarly, neutrophils allowed to spread on glass were stiffer than neutrophils on poly(HEMA). Although neutrophils on poly(HEMA)-coated glass change shape when stimulated, phase-contrast light microscopic studies revealed no tendency to spread. Accordingly, we believe that the mechanical properties of the cells reported here reflect the majority of cells in suspension, and thus of cells circulating in the bloodstream. For our measurements, cover slips were coated with 100 μl of a solution of poly(HEMA) (15 μg/ml) (Aldrich) in ethanol and allowed to dry for 3 hours at 37°C.
34. New Zealand White rabbits, weighing 2.6 to 3.5 kg, were anesthetized with xylazine (2 mg/kg) and ketamine (30 mg/kg) administered intramuscularly. Labeled cells were infused through 23-gauge needles inserted into the marginal ear vein. Anesthesia was maintained by intramuscular injections of ketamine (15 mg/kg) at hourly intervals.
35. These studies were carried out in part in the F. L. Bryant, Jr., Research Laboratory for the Study of Mechanisms of Lung Disease. Supported by grants HL-27353, HL-40784, HL-36577, GM-27160, and GM-38838 from the NIH and DMB-8610636 from the NSF. G.P.D. is supported in part by NIH Clinical Investigator Award HL-02039 and a Medical Research Council of Canada Research Fellowship. G.S.W. has been the recipient of an American Heart Association Clinician-Scientist Award with funds provided in part by the Colorado Heart Association and by an RJR-Nabisco Research Scholars Award. We thank P. M. Henson for critical reading of the manuscript, G. I. Zahalak for helpful discussion, B. McConnaughey for design and maintenance of the cell poker, and J. LaBreque for help with statistical analysis. MAb 60.3 directed against the CD11-CD18 complex was a gift of J. Harlan and P. Beatty.

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Inhibitors of Angiotensin-Converting Enzyme Prevent Myointimal Proliferation After Vascular Injury

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The role of a local angiotensin system in the vascular response to arterial injury was investigated by administering the angiotensin-converting enzyme (CE) inhibitor cilazapril to normotensive rats in which the left carotid artery was subjected to endothelial denudation and injury by balloon catheterization. In control animals, by 14 days after balloon injury, the processes of smooth muscle cell (SMC) proliferation, migration of SMCs from the media to the intima, and synthesis of extracellular matrix produced marked thickening of the intima, with reduction of the cross-sectional area of the lumen. However, in animals that received continuous treatment with the CE inhibitor, neointima formation was decreased (by about 80 percent), and lumen integrity was preserved. Thus, the angiotensin-converting enzyme may participate in modulating the proliferative response of the vascular wall after arterial injury, and inhibition of this enzyme may have therapeutic applications to prevent the proliferative lesions that occur after coronary angioplasty and vascular surgery.

SMOOTH MUSCLE CELL (SMC) proliferation in the intima of muscular arteries and formation of extracellular matrix are major processes that lead to vascular stenosis in arteriosclerosis, after vascular surgery, and after coronary angioplasty (*1*). Although these processes occur in the normal physiology of the vascular wall during development and in response to vascular injury, in excess they may cause significant morbidity (*2*). After endothelial denudation and injury to the arterial wall, the proliferative response in the intima (Fig. 1, A and B) is the result of normally quiescent SMCs migrating from the media to the intima, proliferating both in the media and in the intima (*3*), and producing extracellular matrix.

Several organs contain local angiotensin systems. Both the production of angiotensin II (AII) and its interaction with specific AII receptors occur in these tissues, apparently independently of the plasma renin-angiotensin system (*4*). Angiotensin-converting enzyme (CE) is a membrane-bound enzyme present in the walls of large vessels (both arteries and veins) (*5*), and medial SMCs have high numbers of specific AII receptors (*6*). In addition, treatment of hypertensive animals with inhibitors of CE reduces the medial hypertrophy of muscular arteries that results from chronic hypertension (*7*). We postulated that a local angiotensin system may participate in regulation of the vascular response to arterial injury and investigated the role of endogenous AII production by examining the effects of the specific long-acting CE inhibitor cilazapril (*8*) in an animal model of the proliferative response to arterial injury. This inhibitor was selected because it lowered blood pressure over a 24-hour period and reduced the medial hypertrophy of hypertensive rats (*8*).

Endothelial denudation and vascular injury were achieved in the left common carotid artery of 4-month-old male normotensive rats (*9*) (400 to 500 g) as described (*3*). A balloon catheter (2 French Fogarty, Edwards Laboratories, Santa Anna, California) was passed through the external carotid into the aorta; the balloon was inflated with sufficient water to distend the common carotid and was then pulled back to the external carotid. This procedure was repeated three times and then the catheter was removed. Complete denudation of the endothelium was achieved throughout the common carotid, with some injury to medial SMCs, as assessed by morphological examination of random controls 24 hours after the procedure. The rate of intimal thickening in the rat carotid in response to balloon injury slows considerably after 14 days (*10*). Therefore, at 14 days the animals were anesthetized and perfusion-fixed as described (*11*), with the modification that 2.5% glutaraldehyde and 90-mmHg perfusion pressure were used. Carotid arteries were isolated from adherent tissue and embedded in Epon 812. Semithin sections (1 μm) were stained with toluidine blue and basic fuchsin and processed for morphometric evaluation (*3*). Sections from the middle fifth of the carotid were analyzed, with the right carotid serving as a control for the balloon-catheterized left carotid. Rats were selected randomly to be given either placebo or cilazapril (10 mg/kg per day, mixed with normal food). Animals were coded so that operation and analysis

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