Patients With Acute Coronary Syndrome Show Oligoclonal T-Cell Recruitment Within Unstable Plaque
Evidence for a Local, Intracoronary Immunologic Mechanism

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Background—Recent studies indicate that T-cell activation may play an important role in the pathophysiology of acute coronary syndromes (ACS). However, although those studies detected T-cell expansion in peripheral blood cells, demonstration of specific T-cell expansion within the plaque of patients with ACS is lacking. The present study aims to address whether a specific, immune-driven T-lymphocyte recruitment occurs within the unstable plaque of patients with ACS.

Methods and Results—We simultaneously examined the T-cell repertoire using CDR3 size analysis both in coronary plaques (obtained by directional atherectomy) and in peripheral blood of patients with either ACS (n=110) or chronic stable angina (n=10). Unstable plaques showed a 10-fold increase in T-cell content by quantitative PCR. Using spectratyping analysis, we found several specific T-cell clonotype expansions only in unstable plaque from each patient with ACS, indicating a specific, antigen-driven recruitment of T cells within unstable lesions.

Conclusions—For the first time, T-cell repertoire was investigated directly into coronary plaques; using this approach, we demonstrate that coronary plaque instability in the setting of ACS is associated with immune-driven T-cell recruitment, specifically within the plaque. (Circulation. 2006;113:640-646.)

Key Words: immune system • inflammation • lymphocytes • plaque

Concepts on the pathophysiology of acute coronary syndromes (ACS) have recently undergone considerable evolution, focusing our attention on plaque biology rather than on hemodynamic factors. Indeed, it currently is believed that plaque rupture/erosion, with the consequent superimposed thrombosis, represents a key event causing the sudden conversion of coronary syndromes from chronic to acute.1 In this respect, recent data suggest that immunocompetent, cell-derived inflammation may play an important role in the pathophysiology of plaque rupture.2–4 Thus, to date, the prevailing hypothesis considers inflammation a key event in the pathophysiology of plaque rupture, with macrophages possibly being major effector cells in this process, through the release of matrix-degrading enzymes such as matrix metalloproteinases. Because T cells, through cell-to-cell contact and production and release of cytokines, are the most powerful regulators of macrophage activity,5 T-cell activation has been proposed to represent an important mechanism in the pathophysiology of plaque complication.6 Indeed, it has been reported that lymphocytes from peripheral blood of patients with ACS are characterized by clonal expansions of T cells6–8; these expansions have been attributed to an ongoing antigenic stimulus possibly related to chronic infection.6–8 Indirect evidence of this theory is the finding of a T-cell response against pathogens such as Chlamydia pneumoniae, Helicobacter pylori, and cytomegalovirus in patients with ACS.9,10

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However, the dynamics of T-cell response within the plaque are still poorly understood. In particular, it is unclear whether the inflammation observed in the culprit lesion of patients with ACS is invariably the consequence of a widespread systemic inflammatory status or whether the T-cell...
response is due to a predominantly local, antigen-driven phenomenon. To assess this specific issue, we took advantage of the opportunity afforded by directional coronary atherectomy (DCA) to obtain specimens of coronary plaques from patients with ACS. Using this approach, we were able to study the repertoire of T cells recovered directly from within coronary plaques of patients with ACS by analyzing the region of the T-cell receptor (TCR) critical for antigen recognition (CDR3). Results were compared with those obtained in cells from peripheral blood of the same patients and with cells harvested from coronary plaques of patients with stable angina (SA).

Methods

Patient Population

Human atherosclerotic plaques were obtained from patients with coronary artery disease undergoing DCA. Group 1 included patients with ACS (n=11), defined as typical chest pain at rest occurring <48 hours from hospital admission or ECG changes suggesting myocardial ischemia with or without increase in serum markers of myocardial damage; group 2 comprised patients with chronic SA (n=10) undergoing elective DCA as a revascularization procedure. The local ethics committee approved the experimental protocol, and all patients gave informed consent to participate in the study.

To reduce possible confounding factors, we excluded patients with recent infectious disease, fever, erythrocyte sedimentation rate >20 mm/h, immunosuppressive drug therapy, immunologic disorders, neoplastic disease, or recent (<6 months) major trauma, surgery, or coronary revascularization. Patients also were excluded if their coronary anatomy was not suitable for DCA (tortuous or small vessels, distal lesions), if they had an acute coronary syndrome <1 year before entering the study, or if they showed hemodynamic instability. Thirty consecutive patients met entry criteria and underwent DCA; a sufficient amount of plaque tissue (30 to 40 mg) was obtained in 20. One single plaque was obtained from each patient except 1 patient who underwent DCA of both an unstable and a stable lesion; thus, the final number of plaques undergoing spectratyping analysis was 10 for SA and 11 for ACS patients. In the patient who underwent DCA of 2 lesions, the unstable plaque was identified on the basis of the site of ECG changes and echocardiographic abnormalities, as well as on the angiographic appearance of the lesions.

No significant differences were observed concerning demographic, clinical, and angiographic characteristics of patients in either group (the Table).

Tissue and Blood Sample Collection and Preparation of cDNAs and cDNAs

Immediately after DCA, plaques were frozen and stored in liquid nitrogen until use. Then, 10 mL peripheral blood was collected in EDTA; leukocytes were isolated on a Ficoll gradient; and total RNA was extracted by a single-step method using Trizol according to the manufacturer’s instructions. Stable and unstable plaques were homogenized, and RNA and cDNA were prepared as previously described.11-15 Measurements were performed by investigators unaware of the raw data using reference curves obtained by amplifying for GAPDH.

Clinical Characteristics of the Study Patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SA Patients (n=10)</th>
<th>ACS Patients (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>62±8</td>
<td>59±11</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>7 (70)</td>
<td>7 (70)</td>
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<tr>
<td>Diseased vessels, n</td>
<td>1.7±0.5</td>
<td>1.3±0.5</td>
</tr>
<tr>
<td>DCA vessel</td>
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<td></td>
</tr>
<tr>
<td>LAD</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>RCA</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>LCoX</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Major risk factors, n (%)</td>
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<td></td>
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<tr>
<td>Hypercholesterolemia</td>
<td>4 (40)</td>
<td>5 (50)</td>
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<td>Hypertension</td>
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<td>4 (40)</td>
</tr>
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<td>Diabetes</td>
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<td>2 (18)</td>
</tr>
<tr>
<td>Smoking</td>
<td>3 (30)</td>
<td>5 (50)</td>
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<tr>
<td>Medications, n (%)</td>
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<td>10 (100)</td>
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<td>9 (90)</td>
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<tr>
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<td>10 (100)</td>
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<td>9 (90)</td>
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<td>8 (80)</td>
<td>6 (60)</td>
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<td>ACE inhibitors</td>
<td>4 (40)</td>
<td>4 (40)</td>
</tr>
<tr>
<td>Statins</td>
<td>6 (60)</td>
<td>4 (40)</td>
</tr>
</tbody>
</table>

LAD indicates left anterior descending coronary artery; RCA, right coronary artery; and LCoX, left circumflex coronary artery. Data are expressed as mean±SD. Differences among groups were tested by χ²-analysis.

Clinical Characteristics of the Study Patients

T-Cell Repertoire Analysis

T-cell repertoire was studied by CDR3 length analysis, a technique referred to as spectratyping.11 The TCR is a heterodimer expressed on the cell membrane of T lymphocytes and is formed by α/β or γ/δ chains, which are organized, like the Ig chains, in the CDR1, CDR2, and CDR3 regions.16,17 The CDR3 region is critical for specific antigen recognition.12-14 More than 95% of circulating T cells express α/β TCR.16 Genes coding for TCR proteins undergo rearrangements between V-D-J and C segments, as for immunoglobulins; each VDJ rearrangement (corresponding to CDR3 regions) is unique and defines a cell clonotype.11-14 All cDNAs from either peripheral blood or plaques recovered after DCA were titrated by quantitative PCR for total TCR message and then amplified for all the 25 TCR-β families known by using 1 primer for each β-chain gene (BV) family and 1 common primer for C gene segment.11-15 After amplification, the single PCR products were denatured and run in a fluorescence-based DNA sequencer (Applied Biosystems). Each PCR product amplifies for all the CDR3 lengths in each β-chain family and is visualized as a series of peaks using Genescan Software (Applied Biosystems). With this analysis, each TCR family runs as a series of bands separated by 3 bp with a symmetric form of family-specific spectratype distribution. Deviations from this distribution such as an excess of a particular CDR3 length mark the presence of clonal expansions of T cells such those occurring during a T-cell response.11-13 Because only 1 β-chain is productively rearranged, using the analysis of β-TCR repertoire, we may follow the dynamics of T-cell clonotypes in vivo.12,13 In addition, analysis of CDR3 length also provides information concerning the region that is critical for antigen recognition and dynamics of the T-cell responses.11-19

Quantification of the total TCR message, as well as titration of single cDNAs, was performed by amplifying the message for a constant portion of the TCR β-chain using primers and cycling derived from published sequences, as previously described in details.20,21

Analysis of Cytokine and HLA Gene Expression

Assessment of HLA gene (class I and II) expression and measurement of γ-IFN and IL-4 were performed by quantitative PCR with standard reagents (human γ-IFN, human IL-4, human HLA-B locus, human HLA-DRB1, Taqman Pre Developed Assays Reagents-PDARS, Applied Biosystems) and cycling according to the supplier in a real-time ABI 7500 (Applied Biosystems). Differences in cDNA templates were corrected, titrating all samples according to a preamplification for GAPDH. mRNA copies were calculated from raw data using reference curves obtained by amplifying for GAPDH.
(endogenous control predesigned assay reagent, Taqman Predeveloped Reagents, Applied Biosystems) in each quantitative PCR experiment. To exclude nonspecific amplification and/or the formation of primer dimers, we also ran control reactions in the absence of target cDNA. All experiments were performed in triplicate. In some plaques, the genomic material obtained was of limited amount; therefore, we could run cytokine analysis in only 7 stable and 7 unstable plaques.

Single-Strand Conformational Polymorphism Analysis
To assess the number of different nucleotide sequences in the major peaks found expanded by spectratyping, we performed single-strand conformational polymorphism (SSCP) analysis. Briefly, PCR products of given TCR BV families displaying a predominant peak by spectratyping were serially diluted until only the predominant peak remained visible by Genescan analysis. PCR product (0.5 μL) was then further diluted in 0.5 μL 0.1% NaSO4/10 mmol/L EDTA and run on a nondenaturing gel (6% w/v acrylamide/10% [vol/vol] glycerol). The results were analyzed by Genescan analysis. Under these conditions, SSCP can resolve the different sequences eventually present in 1 spectratyping peak that have the same CDR3 length by their electrical charge, which is directly related to the nucleotide sequence. Thus, the combination of spectratyping and SSCP allows one to resolve the complexity of T-cell repertoire at a clonal level.

Statistical Analysis
Patient Population
Differences in the demographic variables between the 2 groups were tested by χ² analysis.

TCR Analysis
Differences in total TCR mRNA in stable and unstable plaques were tested by the Student t test for unpaired observations. For each plaque, we assigned a score to each TCR BV family, which we called the TCR skewing score, S, as follows: (1) if a TCR signal is very low, then S=0; (2) if a TCR signal appears to be symmetric, then S=1; (3) if a TCR signal appears to be skewed to the left or right, then S=2; and (4) if a TCR signal exhibits a single dominant peak, then S=3. Next, for each plaque, we summed scores for all BV families to obtain a total individual TCR score. The differences in TCR scores between unstable and stable plaques were tested by the Student t test for unpaired observations.

Cytokine and HLA Gene Expression Analysis
Differences in TCR cytokine and HLA gene expression between groups were tested by the Student t test for unpaired observations. All data are expressed as mean±SD.

Results
Total T-Cell Infiltration in Unstable Versus Stable Lesions
To quantify the T-cell infiltrate in the plaques, we performed quantitative PCR for the β-chain of the TCR. The amount of total TCR mRNA was ~10-fold higher in unstable lesions compared with that found in stable lesions, 76,000±3600 versus 7800±980 mRNA copies, respectively (P<0.0001), indicating that unstable plaques contain much more abundant and activated T-cells than their stable counterparts.

Analysis of T-Cell Repertoire in Stable and Unstable Plaques
Figure 1 shows an example of spectratyping analysis covering most of the TCR BV families in 2 typical patients: 1 affected by stable coronary artery disease and 1 with ACS. As predicted, the stable lesion shows an overall low amount of TCR message: in this case, the infiltrate detected by spectratyping had an overall normal T-cell repertoire, as indicated by its symmetric form of family-specific spectratype distribution (Figure 1A). In this patient, peripheral blood also had a normal TCR repertoire, as it appears from its normal distribution (Figure 1A). Similar results of an overall normal T-cell repertoire were found in each SA plaque tested (see below and Figure 2B).

In contrast, unstable plaques were characterized by a highly perturbed T-cell repertoire visualized in this analysis as a severe skewing of most of the TCR BV families, with spectratyping losing the normal symmetric form of family-specific spectratype distribution (Figure 1B). In some instances, distribution was markedly altered by the appearance of a major dominant peak (eg, BV 1, 5.3, 15, 17). This behavior was consistently observed in all 11 patients affected by ACS (Figure 2B). As for peripheral blood, a skewing of distribution of 3 of 25 families of T-cell repertoire was detected in the patient with ACS illustrated in Figure 1B. Similar minor alterations in T-cell repertoire were detected in peripheral blood of 5 of 11 patients with ACS. It has to be stressed that the skewing of the T-cell repertoire observed in ACS patients is present almost exclusively in the plaques; as indicated in Figure 1, most of the profiles of TCR BV families found altered in the unstable lesions have a normal symmetric form of family-specific Spectratype distribution in the peripheral blood.

The overall data for all TCR BV families tested in plaque specimens from each individual patient are visually summarized in Figure 2A. It can easily be appreciated that all unstable plaques have a higher TCR skewing score, indicating a severe perturbation of most of the TCR BV families compared with stable plaques: 42.5±1.5 versus 23.4±1.2 in ACS and SA patients, respectively; P<0.0001 by t test analysis (Figure 2B).

SSCP Analysis
As shown in Figure 1, in patients with ACS, some perturbation of spectratyping profile also may occur in the peripheral blood. Specifically, CDR3 lengths may apparently show a similar distribution between plaque specimens and peripheral blood. To further characterize the oligoclonal T-cell repertoire found in unstable plaques with respect to that observed in peripheral blood, we performed SSCP analysis on the major peaks of TCR BV families found expanded, focusing our attention on those families that apparently had a similar profile both in the plaque and in the peripheral blood. Figure 3 shows SSCP analysis performed on BV1 and BV 5.3 of the patient whose spectratype analysis is detailed in Figure 1B. In both cases, SSCP analysis revealed that the predominant peak at spectratype actually exhibited a single dominant peak with a normal symmetric form of family-specific spectratype distribution (Figure 3). It has to be stressed that the skewing of most of the TCR BV families, with spectratyping losing the normal symmetric form of family-specific spectratype distribution (Figure 1B). In some instances, distribution was markedly altered by the appearance of a major dominant peak (eg, BV 1, 5.3, 15, 17). This behavior was consistently observed in all 11 patients affected by ACS (Figure 2B). As for peripheral blood, a skewing of distribution of 3 of 25 families of T-cell repertoire was detected in the patient with ACS illustrated in Figure 1B. Similar minor alterations in T-cell repertoire were detected in peripheral blood of 5 of 11 patients with ACS. It has to be stressed that the skewing of the T-cell repertoire observed in ACS patients is present almost exclusively in the plaques; as indicated in Figure 1, most of the profiles of TCR BV families found altered in the unstable lesions have a normal symmetric form of family-specific Spectratype distribution in the peripheral blood.

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clinical presentation of the disease (non–ST-segment elevation myocardial infarction), and a stable plaque localized in the right coronary artery. It is interesting to note that severe skewing of the T-cell repertoire again was observed only in the unstable plaque, whereas the stable plaque showed a normal T-cell repertoire (Figure 4).

Analysis of Cytokine and HLA Gene Expression
In unstable plaques, a marked upregulation of both HLA class II molecules and γ-IFN could be observed, whereas IL-4 levels did not change significantly with respect to stable plaques (Figure 5), indicating that T-cell recognition and the consequent Th1 response may have a critical role in the outcome of unstable lesions.

Discussion
Several mechanisms have been proposed to be involved in the pathophysiology of plaque rupture, including mechanical causes (eg, injury by high shear stress rate, fibrous cap fatigue, or damage of the vasa vasorum), resulting in intraplaque hemorrhage, and biological causes (eg, inflammation of the plaque), resulting in apoptosis of smooth muscle cells, macrophage infiltration, release of matrix metalloproteinases, and degradation of extracellular matrix. Overall, these mechanisms may contribute to weakening of the plaque.1–4

In the present study, we exploited the opportunity afforded by DCA to directly investigate T-cell dynamics in plaque specimens obtained in vivo from patients with ACS and to verify whether any T-cell expansions possibly found in material harvested from the plaque also were detected in lymphocytes simultaneously isolated from peripheral blood. Using this approach, we could directly document for the first time that a specific, immune-driven inflammation takes place within the unstable plaque of patients with ACS, as indicated by the following lines of evidence. First, unstable plaques show much greater T-cell infiltration than plaques from patients with stable coronary artery disease. Second, TCR repertoire within the unstable plaques is skewed in all patients with ACS, whereas only a minor derangement, limited to a few BV families, could be observed in a minority of patients with SA. Finally, and perhaps most importantly, simultaneous investigation of T-cell repertoire in plaques and peripheral blood has allowed us to demonstrate that in most patients a specific T-cell expansion occurs exclusively only within the unstable plaques, not in the peripheral blood.

It is known that in patients with fatal myocardial infarction, the site of acute plaque rupture is marked by an inflammatory response characterized by invasion of macrophages, lymphocytes, and activation of smooth muscle cells expressing HLA class II antigens,23–26 suggesting that an acute, immune-mediated inflammatory reaction may occur on “cross-talking” between different cell types. It is well known that macrophages can be activated by T lymphocytes4,5; therefore, T-cell activation has been proposed to represent an important mechanism in the
pathophysiology of ACS. In this respect, studies done on cadavers have documented local (intracoronary) inflammation in patients with ACS and T-cell expansion in the plaque. It also has been reported that patients with ACS are characterized by clonal expansions of T cells in peripheral blood; the presence of a widespread inflammatory reaction in patients with acute myocardial infarction has also been reported. Other studies, mostly using T cells obtained from peripheral blood of patients with ACS, aimed at characterizing specific T-cell subsets or at obtaining cultures of T cells stimulated with plaque homogenates supplemented with a variety of growth factors. Thus, although those studies have been of pivotal importance because they have shifted the focus on T-cell response and overall on immunologic factors as potentially important mechanisms of coronary plaque unstabilization/rupture, no direct information of T-cell dynamics within plaque can be derived because coronary plaques were not tested. As for investigations conducted after autopsy, they have the limitation that plaque biology might have been affected by the time elapsed before harvesting and by the lack of concomitant assessment of T cells in peripheral blood. Therefore, at the moment, the dynamics of T-cell response within the plaque in patients with ACS are largely unknown, as is the occurrence of a direct relationship between systemic immunologic activation (as detected in peripheral blood) and intraplaque events. To firmly establish a role for intraplaque T cells in ACS and to understand its relationship with systemic inflammation, it is important to investigate T-cell behavior directly within plaques in a strict temporal relationship with the ischemic episode and to simultaneously check for the occurrence of similar T-cell expansions in peripheral blood.

These requirements were met by the experimental design of the present study, which for the first time directly demonstrates that ACS may be driven by an inflammatory, antigen-dependent event that may take place locally; thus, systemic inflammation, although important, is not an obligatory requirement. Further support for this hypothesis is provided by the observation that the patient who underwent removal of 2 lesions showed a normal T-cell repertoire in peripheral blood and in the stable plaque, whereas profound skewing was found in the unstable lesion taken on the same occasion.

It is interesting to note that similar patterns of T-cell repertoire were found in some patients with ACS, eg, the behavior of TCR...
BV families 1 and 12 (see Figure 2A), suggesting that there could be conserved CDR3 motif(s) that may indicate a selective expansion by a given antigen. However, more detailed studies are needed to clarify these issues.

Finally, the analysis of cytokine production and HLA expression showed a marked upregulation of HLA class II and γ-IFN in unstable plaques. Although it is likely that such an upregulation can occur in several cell types within the unstable lesion, we would like to stress that HLA class II increases on activated T cells and on other cells when they acquire properties related to antigen presentation. These data, together with the data on the

behavior of T-cell repertoires, strongly suggest that a specific T-cell response against antigen(s) is occurring exclusively in the unstable plaque.

A potential limitation of the present study is that the observed changes in the TCR repertoire between stable and unstable plaques could reflect a difference in terms of plaque harvesting from different areas of the coronary artery in the 2 groups of patients. Although possible, we believe that this hypothesis is indeed very unlikely for the following reasons. First, the presence of a relatively large number of patients in each group should have reduced to a minimum, or even

Figure 4. Spectratyping analysis of 1 stable and 1 unstable plaque obtained from a single patient. This patient required hospitalization because of an ACS, the culprit lesion responsible for which was located in the left anterior descending artery (angiogram on the left); a stable lesion was also present in the right coronary artery (angiogram on the right). Skewing of the normal TCR repertoire again was observed only in the unstable plaque, providing further support to the notion that T-cell expansion is a predominantly local phenomenon taking place within the unstable lesion. As in Figure 1, insets show an enlarged view to demonstrate that in the stable plaque, even in presence of low signal, T-cell repertoire had a normal, symmetric form of family-specific spectratype distribution.

Figure 5. Expression of γ-IFN, HLA class I and II, and IL-4 expression levels in stable and unstable plaques. γ-IFN and HLA class II mRNA are markedly upregulated in unstable plaques. Data are expressed as mean±SD (*P<0.001 vs corresponding value in stable plaques by the Student t test for unpaired observations) (n=7 in each group).
eliminated, the problem of plaque harvesting from different areas of the coronary artery in the 2 groups of patients just by chance. Second, it should be emphasized that because of the design of the atherectomy catheter, DCA allows removal only of plaque material; it leaves intact the normal segments of the vessel.

Conclusions
The present study directly demonstrates for the first time the existence of an antigen-driven inflammatory reaction present in the culprit lesions of patients with ACS, which may occur independently of systemic immunologic activation. Further studies aimed at identifying the antigen(s) responsible for the activation of T cells within the lesions are warranted.

Acknowledgments
This work was supported in part by the following research grants: PRIN 2003, L. 5 from Regione Campania, Centro di Eccellenza per le Malattie Cardiovascolari, and Centro di Competenza GEAR. We wish to thank Dr Elena N. Naumova, PhD, from the Department of Family Medicine and Community Health, Tufts University School of Medicine, Boston, Mass, for her helpful advice in the statistical analysis of the data. The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Disclosures
None.

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Circulation. 2006;113:640-646
doi: 10.1161/CIRCULATIONAHA.105.537712
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

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