Multicentric Inflammation in Epicardial Coronary Arteries of Patients Dying of Acute Myocardial Infarction

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OBJECTIVES
We sought to test the hypothesis of whether inflammatory cell infiltration in patients dying of an acute myocardial infarction (MI) is a multifocal event involving multiple coronary branches.

BACKGROUND
Coronary instability is thought to reflect local disruption of a single vulnerable plaque. However, previous postmortem studies have not addressed the question of whether activation of inflammatory cells, particularly T lymphocytes, is limited to the culprit lesion only or rather diffuse in the coronary circulation.

METHODS
We performed a systematic flow cytometric study in three groups of autopsied patients (group 1 = acute MI; group 2 = old MI; group 3 = no ischemic heart disease). Cell suspensions of enzymatically digested coronary arteries were stained for flow cytometry with CD3, CD68, alpha-smooth muscle actin, and human leukocyte antigen (HLA)-DR antibodies.

RESULTS
The coronary plaques showed: 1) a higher proportion of inflammatory cells in groups 1 and 2 than in group 3; 2) a higher percentage of T lymphocytes in group 1 than in group 2 (11.67 ± 0.70% vs. 5.67 ± 0.74%, p = 0.001) and in group 2 than in group 3 (p = 0.008); and 3) diffuse cell activation in the whole coronary tree of group 1, but not of group 2 subjects.

CONCLUSIONS
Our study suggests that lymphocytes may play a key role in coronary instability by determining activation of various cellular types throughout the coronary circulation. Activated T lymphocytes and their products may well represent a new target in both the treatment and prevention of acute coronary syndromes. (J Am Coll Cardiol 2002;40:1579–88) © 2002 by the American College of Cardiology Foundation

It is well established that acute myocardial infarction (MI) is due to thrombosis at the site of a single disrupted atherosclerotic plaque (1). The complex interplay between factors intrinsic to the plaque and extrinsic events leading to coronary thrombosis is not yet completely understood. Coronary instability is thought to reflect local disruption of a single vulnerable plaque. Yet, in the past few years, several surgical, angiographic, and pathophysiologic studies have suggested the intriguing possibility that coronary instability might develop instead in a multifocal pattern, resulting in the simultaneous presence of multiple unstable plaques in different coronary branches (2–4). Postmortem studies in patients dying of an acute MI have consistently found inflammatory cell infiltration at the site of rupture of the culprit atherosclerotic plaque, thus suggesting that it might play a key role in determining plaque disruption (5). However, previous postmortem studies have not addressed the question of whether activation of inflammatory cells, particularly T lymphocytes, is limited to the culprit lesion only or rather diffuse in the coronary circulation.

To test the hypothesis of whether inflammatory cell activation in patients dying of an acute MI is a multifocal event involving multiple coronary branches, we performed a systematic flow cytometric study using a novel technique we have recently described (6), which provided us with a quantitative measurement of the “inflammatory burden” present in each major coronary artery branch in 30 of 62 consecutive patients who underwent postmortem examination.

METHODS

Study group. Subjects were obtained from a series of 62 consecutive autopsies, according to the following exclusion criteria: age <50 years (n = 3), age >75 years (n = 8), presence of cancer (n = 6), presence of chronic inflammatory diseases (n = 1), multiple myocardial infarctions (n = 3), and interval from death to autopsy >12 h (n = 11). The 30 remaining subjects (20 men and 10 women; age range 55 to 75 years) were enrolled. Autopsies were carried out from 6 to 12 h (median 7.5) after death.

Subjects were allocated to the following three groups (Table 1): 1) group 1 with acute MIs included 16 subjects (11 men and 5 women; age range 55 to 75 years) who died of an acute MI (death occurred within 96 h of chest pain...
onset); 2) group 2 with old MIs included 8 subjects (5 men and 3 women; age range 59 to 72 years) who had a history of acute MI >3 months before death (7 of these patients died of bronchopneumonia and 1 died of peritonitis); and 3) group 3 with no clinical history of ischemic heart disease included 6 subjects (4 men and 2 women; age range 56 to 73 years) who died of bronchopneumonia (n = 5) or melena (n = 1).

**Gross examination of hearts.** The hearts were transversally cut at 1-cm intervals from the apex to base. The myocardium was examined for the presence and extent of infarction. The location of the myocardial infarction was achieved by immersion of a cross section of the heart in a solution of triphenyltetrazolium chloride (TTC) (7). In addition, in all cases, another cross section of the heart was processed for histologic examination, and the infarction was confirmed by light microscopy. The infarction time was determined histologically according to the method of Fishbein et al. (8). We considered the coronary branch supplying the TTC-positive area as an infarct-related artery (IRA).

**Coronary artery preparation.** Coronary artery preparation and subsequent analyses were carried out according to a method recently described by our group (6). Briefly, right, left anterior descending, and circumflex coronary arteries were dissected free of the surrounding adipose tissue and thoroughly washed in buffered saline at pH 7.2 before slicing of the heart. The weight of each sample has been measured before digestion, and no differences were observed between the different tissue samples. The mean weight was 0.88 g (95% confidence interval [CI] 0.79 to 0.97 g) in the acute MI group, 0.87 g (95% CI 0.74 to 1.00 g) in the old MI group, and 0.86 g (95% CI 0.77 to 0.95 g) in the control group (p = 0.73 by analysis of variance [ANOVA]). Coronary arteries were then opened longitudinally and examined to assess the presence of thrombi and atherosclerotic plaques. In each coronary artery branch, a cross section was obtained at the site of the most prominent atherosclerotic plaque; the section was formalin-fixed and paraffin-embedded to verify the degree of preservation of cellular antigens. The IRAs, non-IRAs, and control arteries were subsequently processed separately. The external media layer and the adventitia of each coronary artery branch were carefully peeled off under a dissecting microscope. The remainder of the vessel wall, including the inner media and atherosclerotic plaques, was extensively washed in phosphate-buffered saline (PBS) to remove blood cells adherent to the surface of the plaque, minced into fine pieces, and digested overnight at 37°C with collagenase type I (Sigma Chemical Co., St. Louis, Missouri) at 900 U/ml in Roswell Park Memorial Institute 1640 tissue cultures medium supplemented with 10% fetal calf serum.

The resulting cell suspension that contained all cells initially present in the atherosclerotic plaques and intima (6) was filtered through a 150-mesh nylon net, washed in PBS, and stained for flow cytometry. A total of 2.5 \( \pm 0.65 \times 10^5 \) cells (counted using a manual hemocytometer) was obtained from each sample without significant differences among samples. In the methodologic study we recently published (6), we found that cell death associated with the incubation procedure is negligible (95% cell viability by the trypan blue dye exclusion test), and that the incubation procedure neither affects the cell antigenic properties nor causes detectable cell activation. Of note, after collagenase digestion, the inner media remains undigested, thus avoiding contamination from smooth muscle cells from the arterial wall (6).

**Flow cytometry.** Flow cytometry was performed according to the method recently described by our group (6). Briefly, the following antigens were tested: CD3 for T lymphocytes, CD19 for B lymphocytes, factor VIII for endothelial cells, CD68 for monocytes and macrophages, alpha-smooth muscle actin-1 (SMA) for smooth muscle cells, and human leukocyte antigen (HLA)-DR for activated cells. Because a preliminary study performed in five cases of monoclonal antibodies to CD19 and factor VIII did not demonstrate any significant labeling, monoclonal antibodies to CD3, CD68, SMA, and HLA-DR only were used in this study.

Therefore, each suspension was divided into five aliquots each containing \( 5 \times 10^5 \) cells to assess: 1) unstained cells (negative control); 2) nonspecifically stained cells (mixture of phycoerythrin [PE]/fluorescein isothiocyanate [FITC] secondary conjugated antibodies); 3) CD3/CD68/SMA (double-labeling: CD3 PE-labeled and SMA FITC-labeled monoclonal antibodies); 4) CD68 (FITC-labeled monoclonal antibodies); and 5) HLA-DR (FITC-labeled monoclonal antibodies). Peripheral blood mononuclear cells isolated by Ficoll-Plaque (Pharmacia, Uppsala, Sweden) gradient centrifugation from the peripheral blood of healthy donors were used as positive control samples for lymphocytes and monocytes; smooth muscle cells isolated by elastase digestion from human carotid media were used as positive control samples for smooth muscle cells.

All cell suspensions were analyzed after three washes in PBS with a flow cytometer (Coulter Epics XL, Coulter Corp., Hialeah, Florida) equipped with an air-cooled, 15-mW, argon ion laser operating at 488 nm. The forward scatter, side scatter, and fluorescent intensity and compensation were set using both positive and negative controls. The data were analyzed using the Listmode Analysis of the

### Abbreviations and Acronyms

- **ANOVA** = analysis of variance
- **CI** = confidence interval
- **FITC** = fluorescein isothiocyanate
- **HLA** = human leukocyte antigen
- **IRA** = infarct-related artery
- **MI** = myocardial infarction
- **PBS** = phosphate-buffered saline
- **PE** = phycoerythrin
- **SMA** = smooth muscle actin
- **TTC** = triphenyltetrazolium chloride

November 6, 2002:1579–88

Multicentric Inflammation in Acute MI

Spagnoli et al.
Epics CL software. The amount of each cell population was calculated by counting the positive events after subtracting the nonspecific events falling in the region of the positive control cells (Fig. 1). The whole cell population was obtained by summing the positive events for the CD3, CD68, and alpha-SMA subpopulations and then quantified as percentages.

Cell suspensions contained large amounts of debris from the necrotic core. Because debris displays an intense and heterogeneous autofluorescence and was particularly sticky for fluorescent molecules, a quality-control panel was developed for both testing the quality of the specimens and setting the side and forward scatters, the intensity of fluorescence, and the color compensation. The quality-control panel included peripheral blood mononuclear cells, cultured human smooth muscle cells from the tunica media, and cell plaque samples stained with an irrelevant antibody or with secondary-conjugated antibodies omitting the primary antibody (6) (Fig. 1). To evaluate whether cells from intramural or undetected residual thrombi adherent to the arterial wall could modify cell composition, in a pilot study of three patients not enrolled in this study who died of an acute MI, we compared the cell composition in coronary

### Table 1. Demographic and Clinical Features of Subjects Enrolled in the Study

<table>
<thead>
<tr>
<th>Cases</th>
<th>Myocardium</th>
<th>Gender</th>
<th>Age (yrs)</th>
<th>IRA</th>
<th>Time*</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>Posterolateral wall: yellow-brown, soft necrotic area, with a hyperemic border</td>
<td>M</td>
<td>71</td>
<td>LCx</td>
<td>48 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#2</td>
<td>Posterolateral wall, interventricular septum: yellow area with hemorrhagic foci, heart rupture</td>
<td>M</td>
<td>54</td>
<td>RCA</td>
<td>96 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#3</td>
<td>Posterior wall, interventricular septum: heart rupture, necrotic focus, soft hyperemic borders</td>
<td>M</td>
<td>55</td>
<td>RCA</td>
<td>72 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#4</td>
<td>Anterolateral wall: gray-brown area</td>
<td>F</td>
<td>75</td>
<td>LAD</td>
<td>24 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#5</td>
<td>Posterior wall: wide yellow, softened area rimmed by hyperemic border</td>
<td>M</td>
<td>56</td>
<td>RCA</td>
<td>48 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#6</td>
<td>Anterior wall: pale gray-brown, softened area</td>
<td>M</td>
<td>64</td>
<td>LAD</td>
<td>24 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#7</td>
<td>Posterior wall: pale, soft area with hyperemic border</td>
<td>F</td>
<td>69</td>
<td>RCA</td>
<td>48 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#8</td>
<td>Posterolateral wall: yellow, softened area rimmed by hyperemic border</td>
<td>M</td>
<td>59</td>
<td>LCx</td>
<td>48 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#9</td>
<td>Posterolateral wall: yellow, softened necrotic area with hyperemic border</td>
<td>F</td>
<td>68</td>
<td>LCx</td>
<td>48 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#10</td>
<td>Posterior wall, interventricular septum: yellow-brown, softened area with hyperemic border</td>
<td>M</td>
<td>69</td>
<td>RCA</td>
<td>48 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#11</td>
<td>Anterior wall: myocardial scar, acute necrotic area</td>
<td>M</td>
<td>67</td>
<td>LAD</td>
<td>48 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#12</td>
<td>Anterolateral wall: myocardial scar, yellow-brown, soft necrotic focus with hyperemic border</td>
<td>F</td>
<td>56</td>
<td>LAD</td>
<td>48 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#13</td>
<td>Anterior wall: myocardial scar, yellow-brown, soft necrotic focus</td>
<td>M</td>
<td>64</td>
<td>LAD</td>
<td>48 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#14</td>
<td>Anterolateral wall: myocardial scar, yellow-brown, soft necrotic focus with hyperemic border</td>
<td>F</td>
<td>67</td>
<td>RCA</td>
<td>96 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#15</td>
<td>Anterior wall: myocardial scar, yellow, soft necrotic focus with hyperemic border</td>
<td>M</td>
<td>72</td>
<td>LAD</td>
<td>24 h</td>
<td>AMI</td>
</tr>
<tr>
<td>#16</td>
<td>Posterolateral wall: yellow-brown, soft necrotic focus with irregular hyperemic border</td>
<td>M</td>
<td>76</td>
<td>LAD</td>
<td>48 h</td>
<td>AMI</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cases</th>
<th>Myocardium</th>
<th>Gender</th>
<th>Age (yrs)</th>
<th>IRA</th>
<th>Time*</th>
<th>Cause of Death</th>
</tr>
</thead>
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<tr>
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<td>Anterior wall: fibrous white-gray area 3.5 cm</td>
<td>M</td>
<td>68</td>
<td>LAD</td>
<td>1 year</td>
<td>Peritonitis</td>
</tr>
<tr>
<td>#18</td>
<td>Anterior wall: fibrous white-gray tissue</td>
<td>F</td>
<td>75</td>
<td>LAD</td>
<td>2 years</td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>#19</td>
<td>Posterior wall: fibrous white-gray area 4 cm</td>
<td>M</td>
<td>69</td>
<td>RCA</td>
<td>1 year</td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>#20</td>
<td>Anterior wall: fibrous white-gray area 3 cm</td>
<td>M</td>
<td>71</td>
<td>LAD</td>
<td>1 year</td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>#21</td>
<td>Anterior wall: fibrous white-gray area 3 cm</td>
<td>M</td>
<td>66</td>
<td>LAD</td>
<td>1 year</td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>#22</td>
<td>Lateral wall: fibrous white-gray area 3.5 cm</td>
<td>F</td>
<td>71</td>
<td>LCx</td>
<td>1 year</td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>#23</td>
<td>Anterior wall: fibrous white-gray area 2.8 cm</td>
<td>F</td>
<td>68</td>
<td>LAD</td>
<td>2 years</td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>#24</td>
<td>Anterior wall: fibrous white-gray area 2.3 cm</td>
<td>M</td>
<td>65</td>
<td>LAD</td>
<td>1 year</td>
<td>Bronchopneumonia</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cases</th>
<th>Myocardium</th>
<th>Gender</th>
<th>Age (yrs)</th>
<th>IRA</th>
<th>Time*</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>#25</td>
<td>No pathologic lesions on myocardium</td>
<td>M</td>
<td>65</td>
<td></td>
<td></td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>#26</td>
<td>No pathologic lesions on myocardium</td>
<td>M</td>
<td>74</td>
<td></td>
<td></td>
<td>Intestinal hemorrhage</td>
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<td>#27</td>
<td>No pathologic lesions on myocardium</td>
<td>F</td>
<td>69</td>
<td></td>
<td></td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>#28</td>
<td>No pathologic lesions on myocardium</td>
<td>M</td>
<td>59</td>
<td></td>
<td></td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>#29</td>
<td>No pathologic lesions on myocardium</td>
<td>F</td>
<td>67</td>
<td></td>
<td></td>
<td>Bronchopneumonia</td>
</tr>
<tr>
<td>#30</td>
<td>No pathologic lesions on myocardium</td>
<td>M</td>
<td>65</td>
<td></td>
<td></td>
<td>Bronchopneumonia</td>
</tr>
</tbody>
</table>

*Period after myocardial infarction. AMI = acute myocardial infarction; IRA = infarct-related artery; LAD = left anterior descending coronary artery; LCx = left circumflex coronary artery; RCA = right coronary artery.
Figure 1. Acquisition and analysis two-color flow cytometry. Panels A, B, C, D, E, and F represent the control set. (A) Monocytes from peripheral blood cells stained with CD68; the region delimits the area in which positive cells are identified. (B) Smooth muscle cells from the normal tunica media stained with alpha-smooth muscle actin (SMA); the region delimits the area in which positive cells are identified. (C) Lymphocytes from peripheral blood cells stained with CD3; the region delimits the area in which positive cells are identified. (D) Phytohemagglutinin (PHA)-activated peripheral blood cells stained with human leukocyte antigen (HLA)-DR; the region delimits the area in which positive cells are identified. (E) Coronary samples stained with control antibody (mixture of fluorescein isothiocyanate (FITC)/phycoerythrin (PE)-conjugated secondary antibodies). (F) A region gate was drawn to delimit the nonspecific stain. (G) Coronary cells stained with CD3. G1 shows single PE-immunostained cells after debris gating according to control settings. G2 shows the results in the positive region after removal of the debris. (H) Coronary cells stained with CD68. H1 shows single PE-immunostained cells after debris gating according to control settings. H2 shows the results in the positive region after removal of the debris. (I) Coronary cells stained with alpha-SMA. I1 shows single FITC-immunostained cells after debris gating according to control settings. I2 shows the results in the positive region after removal of the debris. (J) Coronary cells stained with HLA-DR. J1 shows single PE-immunostained cells after debris gating according to control settings. J2 shows the results in the positive region after removal of the debris.
segments containing thrombus and in the remaining IRA wall. In this pilot study, no difference was found in segments containing or not containing thrombus (CD3+ cells: 15.04% [95% CI 14.18% to 15.90%] vs. 13.07% [95% CI 10.59% to 15.55%], p = NS; CD68+ cells: 27.93% [95% CI 24.57% to 31.29%] vs. 23.64% [95% CI 9.70% to 37.58%], p = NS, respectively), thus proving that undetectable thrombi did not significantly affect cell composition.

**Immunocytochemistry for conventional and confocal microscopy.** To test tissue antigenicity, paraffin sections were incubated with the primary monoclonal antibodies to SMA, CD68, and CD3. Control sections were incubated with a mixture of irrelevant monoclonal reagents with a similar isotype.

Because our flow cytometric protocol did not allow the correct analysis of double-labeled cells, double immunostaining was performed to verify the positivity of plaque cell types for HLA-DR. Sections were first incubated with monoclonal antibodies to CD3, rinsed, and then incubated with biotinylated mouse immunoglobulin G. They were then rinsed again and incubated with antibodies conjugated to streptavidin peroxidase. To enhance the signal, sections were incubated using the Tyramide signal amplification biotin system (NEN Life Science Products, Boston, Massachusetts). Fluorescence was obtained by incubating the sections with a streptavidin–Texas red fluorescent conjugate. After this first reaction, a second reaction with primary antibodies to HLA-DR was induced as described earlier, and fluorescence was obtained with a streptavidin–fluorescein conjugate (i.e., FITC). Specimens were observed with a conventional fluorescent microscope and a confocal microscope. Images were acquired by means of the Noran confocal microscope with a 60×/1.4 NA immersion oil lens. Three D stacks were acquired at a resolution of 0.1 μ in the X, Y, and Z axis.

**Statistical analysis.** One-way ANOVA was used to compare cell immunophenotypes among the three groups: those with an acute MI (group 1, n = 16), old MI (group 2, n = 8), and control subjects with no clinical history of ischemic heart disease (group 3, n = 6). Two-way ANOVA was used to compare cell immunophenotypes between the IRA and non-IRA in groups 1 and 2. This analysis was carried out for the following cell immunophenotypes: CD3+, CD68+, actin+, and HLA-DR+–positive cells. For an F value with a p value <0.05, pair-wise comparisons were carried out using the Student t test with Bonferroni correction. Multiple linear regression analysis was used to evaluate the correlation between HLA-DR+–positive cells and CD3+, CD68+, and SMA-positive cells in each group of patients.

Data were analyzed by using SPSS (Statistical Package for the Social Sciences) software. Data are given as the mean value with the 95% CI. In all analyses, p < 0.05 was considered statistically significant.

**RESULTS**

**Gross examination.** In Group 1, the gross inspection of the three major epicardial coronary artery branches showed severe and diffuse coronary atherosclerosis with calcifications and multiple stenoses. The IRAs were characterized by occlusive thrombosis in two cases, whereas in the remaining cases, nonocclusive mural thrombus was found. In contrast, the non-IRAs did not exhibit any thrombus.

In groups 2 and 3, the three major epicardial coronary arteries were characterized by a variable degree of atherosclerosis and a lack of thrombosis.

**Flow cytometric findings.** During flow cytometry, cell populations were composed of variable proportions of smooth muscle cells, monocytes and macrophages, and T lymphocytes.

Although smooth muscle cells were the prevalent cell type in all specimens, significant differences were found in the percentage of inflammatory cells both among groups and between the IRA and non-IRA (Fig. 2 and 3). Indeed, the percentage of T lymphocytes was higher in group 1 than in group 2 (11.67% [95% CI 10.26% to 13.08%] vs. 5.67% [95% CI 4.15% to 7.20%], p = 0.001 by the Bonferroni test) and was higher in group 2 than in group 3 (5.67% [95% CI 4.15% to 7.20%] vs. 1.71% [95% CI 0.94% to 2.48%], p = 0.008 by the Bonferroni test). Conversely, the percentage of monocytes and macrophages was higher in group 2 than in groups 1 and 3 (39.98% [95% CI 34.42% to 45.54%] vs. 24.85% [95% CI 21.51% to 28.20%] and 19.37% [95% CI 14.18% to 24.54%], p = 0.001 by the Bonferroni test, respectively). Two-way ANOVA has shown that the percentage of T lymphocytes and HLA-DR+–positive cells significantly depended both on the clinical groups (p = 0.0001) and the coronary subset (i.e., IRA vs. non-IRA, p = 0.0001).

The percentage of T lymphocytes was significantly higher in the IRA than in the non-IRA both in group 1 (16.19% [95% CI 13.96% to 18.41%] vs. 9.40% [95% CI 8.18% to 10.63%], p = 0.001 by the Bonferroni test) and group 2 (9.32% [95% CI 6.69% to 11.96%] vs. 3.85% [95% CI 2.69% to 5.01%], p = 0.002 by the Bonferroni test). Conversely, the percentage of monocytes and macrophages was similar in the IRA and non-IRA in both groups 1 and 2 (21.88% [95% CI 16.51% to 27.26%] vs. 26.34% [95% CI 21.99% to 30.68%], p = NS by the Bonferroni test) and 38.75% [95% CI 33.72% to 43.77%] vs. 40.60% [95% CI 32.21% to 48.98%], p = NS by the Bonferroni test, respectively).

The percentage of HLA-DR+–positive cells was higher in group 1 than in group 2 (70.35% [95% CI 53.00% to 87.70%] vs. 39.41% [95% CI 30.89% to 47.92%], p = 0.01 by the Bonferroni test) and was higher in group 2 than in group 3 (39.41% [95% CI 30.89% to 47.92%] vs. 5.06% [95% CI 3.17% to 6.94%], p = 0.001 by the Bonferroni test).

The percentage of HLA-DR+–positive cells was similar in the IRA and non-IRA in group 1 (78.22% [95% CI 65.51% to 90.92%] vs. 66.42% [95% CI 42.17% to 90.66%], p = NS by the Bonferroni test), although it was higher in the IRA than in non-IRA in group 2 (58.19% [95% CI 44.82% to
Figure 2. Distribution of cell immunophenotypes in group 1 (acute myocardial infarction [MI]), group 2 (old MI), and group 3 (no clinical history of ischemic heart disease). One-way analysis of variance showed significant differences ($p = 0.0001$) in the distribution of the various cytotypes and human leukocyte antigen (HLA)-DR–positive cells among the experimental groups. The Bonferroni test showed the following significant differences ($p < 0.01$) for actin (smooth muscle actin [SMA]) (group 1 vs. 2 and 3; group 2 vs. 3), CD3 (group 1 vs. 2 and 3; group 2 vs. 3), CD68 (group 1 vs. 2; group 2 vs. 3), and HLA-DR (group 1 vs. 2 and 3; group 2 vs. 3). Data are presented as the mean value with 95% confidence interval.

Figure 3. Distribution of cell immunophenotypes in the infarct-related artery (IRA) and non-IRA of subjects with an acute myocardial infarction (MI) and those with an old MI. One-way analysis of variance showed significant differences ($p = 0.0001$) in the distribution of CD3+ ($p = 0.0001$), CD68+ ($p = 0.0001$), and human leukocyte antigen (HLA)-DR–positive cells ($p = 0.004$) among the experimental groups. The Bonferroni test showed the following significant differences ($p < 0.05$) for CD3 (a vs. b, c, and d; b vs. d; c vs. d), CD68 (a vs. c and d; b vs. d), and HLA-DR (d vs. a, b, and c). Data are presented as the mean value with 95% confidence interval.
Figure 4. Immunohistochemical staining against human leukocyte antigen (HLA)-DR antigens and flow cytometric acquisition of HLA-DR phycoerythrin-conjugated stain in various experimental groups. **(A)** A coronary section from the infarct-related artery (IRA) (group 1; case no. 1 in Table 1) shows a diffuse positive reaction in the plaque and in the site of cap rupture. **A1** shows the flow cytometric results of the same coronary artery after removal of the debris. **(B)** A coronary section from the non-IRA (same case no. 1) shows a strong positive reaction in the cells through the whole plaque. **B1** shows the flow cytometric results after removal of the debris in the same coronary artery. **(C)** A coronary section from the IRA of a group 2 subject (case no. 22 in Table 1). The positive reaction to HLA-DR is comparable to that in the non-IRA of group 1 (see panels B and Fig. 3). **C1** shows the flow cytometric results of the same coronary artery after removal of the debris. **(D)** A coronary section from group 3 (case no. 30 in Table 1). Only a few cells were positive for the HLA-DR antibody. **D1** shows coronary cells from the same coronary artery.
Multiple linear regression analysis applied to flow cytometric data demonstrated a strong correlation between the percentage of HLA-DR-positive cells and the percentage of T cells ($r = 0.77$, $p = 0.004$) in group 2 only, although no correlation was found between the percentage of HLA-DR-positive cells and the percentage of various cytotypes in groups 1 and 3.

Immunohistochemistry. Cell antigens were well preserved in all samples. Indeed, in situ immunophenotyping showed a strong positivity for CD68, CD3, SMA, and HLA-DR, but not for CD19. Moreover, immunohistochemistry confirmed flow cytometric data showing a similar amount of HLA-DR-positive cells both in the IRA and non-IRA vessels in group 1. The positivity of HLA-DR was diffuse in cell cytotypes and was higher than that found in groups 2 and 3 (Fig. 4), again confirming flow cytometry data.

In situ double immunostaining, examined by confocal microscopy, revealed that in group 1, all T lymphocytes were positive for HLA-DR (Fig. 5).

**DISCUSSION**

The present study, using a novel technique for the quantitative assessment of cellular components of epicardial coronary arteries, has allowed us to demonstrate that coronary atherosclerotic plaques exhibit: 1) a higher proportion of inflammatory cells in patients with an acute or old MI than in control subjects; 2) a higher percentage of T cells in patients with an acute MI than in patients with an old MI; and 3) diffuse cell activation both in the IRA and non-IRA in patients with an acute MI, but not in patients with an old MI.

The demonstration of a higher prevalence of inflammatory cells in patients with overt ischemic heart disease confirms the evidence accumulated over the past few years that atherosclerosis is an inflammatory disease (9) and gives support to the reliability of the novel quantitative approach utilized in this study.

More importantly, our study shows that patients with an acute MI exhibit a different pattern of inflammation as compared with that of patients with stable ischemic heart disease—the former being characterized by a marked increase in the percentage of T lymphocytes. These findings give support to the notion that T-lymphocyte activation and proliferation might play an important role in determining the transition from the chronic to acute phases of ischemic heart disease (5,10–12). Accordingly, Neri Serneri et al. (13) observed a significant and transient increase in activated T lymphocytes in the peripheral blood of patients affected by unstable angina. More recently, Caligiuri et al. (14) found that the antigen receptor repertoire of activated T cells was skewed in 57% of patients with unstable angina, as compared with 23% of patients stable ischemic heart disease, supporting the hypothesis that an antigen-driven immune response may play a role in the pathogenesis of coronary instability. They also found a specific proliferative response to proteins contained in the atherectomy specimens of unstable patients but not stable patients, thus suggesting that the antigenic triggers might be located at the site of the culprit lesion. The notion of an important role of T lymphocytes in the transition from the chronic to acute phases of ischemic heart disease is also supported by the findings of Liuzzo et al. (12), who reported that a subset of patients with unstable angina have expanded unusual CD4+/CD28null T cells that excessively produce interferon-gamma on stimulation. Circulating CD4+/
CD28null cells might infiltrate the coronary plaque and induce inflammation, particularly if stimulatory antigen is locally expressed. Although a response to persistent antigen should be beneficial, expansion of CD4+/CD28null cells appears to be correlated with negative consequences. Indeed, CD28null cells are unable to support B-cell differentiation and antibody production (15), as well as cytolytic abilities.

It is worth noting that in our study, the prevalence of activated HLA-DR-positive cells was higher in subjects with an acute MI than in those with an old MI. More importantly, the prevalence of activated cells was similar in the IRA and non-IRA in the former group, whereas it was higher in the IRA than in non-IRA in the latter group. Furthermore, a strong correlation between T cells and HLA-DR-positive cells was found in patients with an old MI, but not in those with an acute MI. Taken together, these findings suggest that acute MI is associated with activation of T lymphocytes, which, in turn, through the release of interferon-gamma and other cytokines, results in diffuse activation of various cellular types, including smooth muscle cells and monocytes and macrophages in multiple epicardial coronary artery branches. Accordingly, previous studies have shown that acute coronary syndromes are associated with multiple coronary thromboses at postmortem examination (2), with microvascular impairment and transmural activation of inflammatory cells in remote regions, and with enhanced short-term progression of non-culprit stenoses (16,17). More recently, Goldstein et al. (4) found that two-fifths of patients with an acute MI harbor multiple complex coronary plaques, which are associated with adverse clinical outcomes. In aggregate, these pathologic, angiographic, and clinical observations support the concept that plaque instability is not merely a local vascular accident, but probably reflects more generalized pathophysiologic processes with the potential to destabilize atherosclerotic plaques throughout the coronary tree. The triggers responsible for diffuse cell activation throughout the whole coronary circulation of patients with acute coronary syndromes cannot be deduced from the results of our study, but they are likely to be multiple and may have a coronary or even noncoronary location. Regardless of its causes, cell activation in atherosclerotic plaques can cause severe detrimental effects through a variety of different mechanisms, including thrombogenicity due to tissue factor expression, matrix degradation caused by enhanced release of matrix metalloproteinases, and vasoconstriction caused by enhanced release of endothelin. In turn, these mechanisms operating at the site of more vulnerable plaques lead to transient or permanent coronary occlusion and to the acute manifestations of ischemic heart disease.

**Study limitations.** A limitation of our study is the lack of postmortem angiography, which could not be performed because background fluorescence caused by radiopaque media interferes with cytofluorometric analyses. From the present data, it is not possible to assess the distribution of HLA-DR-positive cells among the different cytotypes. This limitation was due to the high background fluorescence caused by debris, which prevented a correct analysis of double staining on the same cell type at flow cytometry (6). Furthermore, another limitation was the lack of pathoanatomic data on the presence of single versus multiple unstable plaques in culprit IRA and non-IRA vessels in patients with and without MI. In fact, morphologic analysis was prevented by the need to utilize most of epicardial coronary arteries for flow cytometric analysis. Finally, this study does not provide information on the triggers of lymphocyte activation in acute MI.

**Conclusions.** Our study suggests that lymphocytes may play a key role in coronary instability by determining activation of various cellular types throughout the coronary circulation. Better knowledge of the triggers of inflammation is probably needed before such inflammation may become a therapeutic target in acute coronary syndromes (18). Our study suggests the intriguing possibility that the trigger of inflammation does not necessarily reside in the culprit atherosclerotic plaque.

**Acknowledgments**

We thank Mr. Alfredo Colantoni and Ms. Angela Ortenzi for their helpful technical assistance.

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