Leukocyte-mediated myocardial reperfusion injury is characterized by the progressive migration and accumulation of polymorphonuclear leukocytes within the myocardium. In this study, we hypothesized that leukocytes normally resident to the myocardium also contribute to myocardial injury in the absence of migration and accumulation of peripheral polymorphonuclear leukocytes. In isolated crystalloid-perfused rat hearts, we found numerous resident cardiac leukocytes that were identified primarily as macrophages and mast cells, the latter staining avidly for peroxidase. When hypoxic perfused hearts (60 minutes, \( n=16 \)) were reoxygenated there was a prompt release of this peroxidase activity, the extent of which correlated closely with the degree of myocardial injury (total creatine kinase release, \( r=0.96 \)). When reoxygenation associated mast cell degranulation was prevented in six additional hypoxic hearts using 10 \( \mu \text{M} \) Lodoxamide Tromethamine, peroxidase release was reduced 7.8-fold (\( p<0.001 \)) and creatine kinase release (injury) was reduced 5.9-fold (\( p<0.001 \)). These results demonstrate that the isolated crystalloid-perfused rat heart is not a leukocyte-free preparation and suggest that mast cells resident to the heart play an important role in acute reoxygenation injury. (Circulation Research 1988;63:1044-1052)

Extensive effort has recently been directed at reduction of the morbidity and mortality of acute myocardial infarction by acute reperfusion of ischemic myocardium.\(^1\)\(^-\)\(^5\) With the exception of brief (10–15 minutes) episodes of coronary artery occlusion, prolonged ischemia followed by reperfusion is invariably accompanied by irreversible myocardial injury.\(^6\) A significant component of this injury is thought to arise from the actual process of reperfusion; moreover, it has been demonstrated that this component of injury can be reduced with various pharmacological interventions.\(^7\)\(^-\)\(^18\)

One such intervention is the inhibition of the migration and activation of peripheral polymorphonuclear leukocytes (PMNL), which has been shown to result in enhanced myocardial salvage in experimental models of myocardial infarction.\(^19\)\(^-\)\(^21\) Presumably, the progressive migration and accumulation of activated PMNL within the heart mediates a delayed form of reperfusion injury (occurring over 1 to 4 hours) by damaging myocardial and endothelial cells,\(^22\)\(^-\)\(^26\) but these cells may also mediate reperfusion injury by causing capillary plugging.\(^19\),\(^27\)\(^,\)\(^28\)

The heart is similar to most other organs in that it has a resident population of leukocytes located within the interstitium.\(^29\),\(^30\) While resident leukocytes also have the potential to mediate significant tissue injury, their role in mediating cardiac reperfusion injury has not been addressed. Thus, the objectives of this study were to 1) characterize the resident myocardial leukocyte population in isolated crystalloid-perfused rat hearts, 2) evaluate the relation between degranulation of these leukocytes (measured by the release of peroxidase) and indexes of cardiac injury (creatine kinase release) when these hearts were first made hypoxic and then reoxygenated, and 3) explore the protective effects of pharmacological inhibition of leukocyte degranulation with Lodoxamide Tromethamine in the same model. The results of this study indicate that resident myocardial mast cells mediate a significant component of myocardial reoxygenation injury.
Materials and Methods

Heart Perfusion

Reoxygenation injury was studied with an isolated hypoxic perfused heart model. Although this model does not compare exactly with ischemia and reperfusion, it is well suited for study of the myocardial damage resulting from the reintroduction of oxygen. Tissue injury is typically produced in this model by perfusion of the heart for 40 to 90 minutes with hypoxic perfusate followed by a change to normoxic perfusate to simulate reperfusion. All animal experiments were performed within the guidelines established by the National Institutes of Health for animal research under a protocol approved by the Columbia University Animal Care Division. Male Wistar rats with an average weight of 300–350 g were used. The animals were fasted overnight and injected with heparin (1,000 units/kg i.p.) 30 minutes before induction of anesthesia. Animals were anesthetized with ketamine (100 mg/ml) containing 4% acemopramine at a dose of 1–2 ml/kg i.p. Following thoracotomy the hearts were quickly excised and arrested in iced isoosmotic saline containing 20 mM KCl. Hearts were then rapidly cannulated and perfused via the aorta with a nonrecirculating Langendorf perfusion apparatus at a constant pressure of 80 mm Hg.

The Krebs-Henseleit perfusate, maintained at 37° C, contained the following concentrations (mM) of solutes: KCl 4.7, CaCl\textsubscript{2} 2.5, KH\textsubscript{2}PO\textsubscript{4} 1.2, NaCl 118, NaHCO\textsubscript{3} 25, and MgSO\textsubscript{4} 1.2. There were three phases of perfusion: The first phase (prehypoxic, experiment time from 0 to 35 minutes) consisted of 35 minutes of aerobic perfusion during which time formed blood elements were washed out of the vascular system. During the prehypoxic phase the perfusate contained 4 mM glucose (without insulin) and was bubbled with 95% \textsubscript{2}\textsubscript{O}-5% \textsubscript{2}\textsubscript{CO}. The second perfusion phase followed the prehypoxic phase and consisted of 60 minutes of hypoxic perfusion (experiment time from 35 minutes to 95 minutes). During this perfusion phase the perfusate contained no glucose and was bubbled with 95% \textsubscript{2}\textsubscript{O}-5% \textsubscript{2}\textsubscript{CO}. Glucose markedly attenuates myocardial damage if it is present during the hypoxia phase, and thus was excluded during this time. The third perfusion phase was an 18-minute reoxygenation period (experiment time from 95 to 113 minutes). Perfusate during this last phase was identical to the perfusate used during the hypoxic phase except that it was oxygenated with 95% \textsubscript{2}\textsubscript{O}-5% \textsubscript{2}\textsubscript{CO}.

At the end of each experiment hearts were removed from the apparatus, blotted dry, the atria and great vessels were removed, and the whole heart was weighed. A 200–300 mg sample of myocardium was removed, weighed, and then placed in a vacuum oven at 80° C for 48 hours before reweighing for determination of wet weight-to-dry weight ratios.

Histochemistry

Hearts used for histology were first excised, perfused for 35 minutes, and then removed for preparation. Portions of myocardium were snap-frozen in dry ice-isopentane, and 5 \textmu m thick sections were obtained. The sections were picked up on glass slides, allowed to thaw, and incubated with a 0.02% solution of dianisidine in phosphate buffered saline, pH 6.3, with 0.06% hydrogen peroxide. The reaction was stopped with azide, and the slides were washed briefly in phosphate buffered saline (PBS), counterstained in hematoxylin, dehydrated through graded alcohols, and coverslipped. Additional frozen sections were counterstained with 1% Toluidine blue, dehydrated, and mounted. Five to 10 sections from each heart were typically studied, from which 100 to 400 fields were systematically examined.

Immunohistochemistry

Immunofluorescence. Frozen sections were prepared as above and fixed briefly in acetone. The sections were rehydrated in PBS, pH 7.25, incubated in normal goat serum for 20 minutes, the excess serum was drained off, and then the sections were incubated for 12 minutes with the appropriately diluted specific fluorescein conjugated anti-rat leukocyte antibody (Accurate Chemical and Scientific Corporation, Westbury, New York). The slides were then thoroughly washed in PBS for an hour, mounted in a 90% glycerol-10% PBS solution, coverslipped, sealed with clear nail polish, and examined with a Zeiss epillillumination fluorescence microscope.

Immunoalkaline phosphatase. Frozen sections were prepared as above, incubated sequentially with normal goat serum, appropriately diluted rabbit anti-rat leukocyte antibody (Accurate Chemical), and alkaline phosphatase-conjugated F(ab')\textsubscript{2} goat anti-rabbit IgG (Accurate Chemical). The reaction product was formed using an alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, California) with added levamisole. Sections were then dehydrated and mounted.

Antibodies. Commercially available polyclonal antisera raised in rabbits were used in the identification of myocardial leukocyte subtypes. These sera were obtained following the specific inoculation of rabbits over 2 weeks with either rat mast cells, PMNL, thrombocytes, lymphocytes, or macrophages. The reported specificity of these antisera were as follows: the anti-rat lymphocyte serum strongly agglutinated rat thymocytes and splenocytes but not PMNL or macrophages; the anti-PMNL serum strongly agglutinated rat PMNL but not other thymocytes or splenocytes; the anti-rat thrombocyte serum strongly agglutinated thrombocytes but not thymocytes or splenocytes; and the anti-rat mast cell serum agglutinated mast cells but not thymocytes or splenocytes.
Creatine Kinase Release: Indicator of Myocardial Damage

To determine the extent of myocardial damage, the total amount of creatine kinase released from the heart was measured. Coronary effluent was collected at 30, 55, 75, and 90 minutes and coronary flow rate measured. During reoxygenation, 3 minute collections of effluent were started at 95, 98, 101, 104, 107, and 110 minutes, and the total volume collected was used to calculate average coronary flow during each interval. Creatine kinase activity in the effluent samples was measured using a commercially available kit (Sigma Chemical, St. Louis, Missouri). Values were corrected for dry heart weight and coronary flow rates and expressed in units per minute per gram dry weight.

Peroxidase Release: Indicator of Leukocyte Degranulation

Leukocyte peroxidase was selected as an index of degranulation as it was easily identified histochemically and was likely to play a role in the injury process. The following assay was used for the spectrophotometric measurement of peroxidase activity: coronary effluent (100 μl), collected as noted above, was combined in immunoassay plates (Nunc, Thomas Scientific) with 0.8 mM o-dianisidine (20 μl), 0.0003% hydrogen peroxide (20 μl), 50 mM NaH₂PO₄ (20 μl), pH 6.3, and water to achieve a final volume of 0.2 ml. After 10 minutes at room temperature, the reaction was stopped with the addition of 20 μl of 0.2% sodium azide. The absorbance at 492 nM was measured with a Multiscan plus enzyme-linked immunosorbent assay plate reader. The extinction coefficient used for the absorbance of oxidized o-dianisidine was 10 mM⁻¹, and results were expressed as units of peroxidase released per minute per gram dry weight, where 1 unit equaled the oxidation of 1 nmole dianisidine per minute. Although it was possible that this assay reacted with a nonspecific myocardial cofluent, and not released peroxidase, this was unlikely because 1) with the same assay for histochemistry, no nonspecific myocardial staining was identified in this study, and 2) eluted peroxidase activity was found to have biochemical characteristics (pH optimum, azide inhibition) typical for peroxidases (see "Results").

Leukocyte Inhibition

To investigate the beneficial effect of inhibition of leukocyte degranulation upon reoxygenation injury the identical perfusion protocol was repeated except that hearts were perfused in the presence of 10 μM Lodoxamide Tromethamine. Lodoxamide (U-42, 585E; a generous gift of the Upjohn Company, Kalamazoo, Michigan) is a potent mast cell stabilizer and markedly attenuates the degranulation of mast cells in response to both specific and nonspecific stimuli. Concentrations in the range of 10 to 100 μM are most effective for inhibiting mast cell degranulation and were found not to interfere with either the peroxidase or creatine kinase assay.

Statistical Analysis

The relation between variables was assessed with a linear regression analysis. Comparison of unpaired data between treatment groups was made with a two-tailed Student’s t test. Comparison of repeated measurements between groups was made using a two way analysis of variance. For all tests the minimal level of significance was p<0.05.

![FIGURE 1. A representative section of perfused rat myocardium incubated with dianisidine to demonstrate peroxidase activity. Granular intracytoplasmic staining in large round interstitial cells is identified by the presence of a light brown precipitate. There is no nonspecific staining for peroxidase activity in myocytes. (Toluidine Blue counterstain, ×300)](http://circres.ahajournals.org/doi/abs/10.1161/01.RES.63.6.1046)
Results

Characterization of the Resident Myocardial Leukocyte Population

The resident population of myocardial leukocytes were first characterized with a panel of specific fluorescent-labeled anti-leukocyte antibodies and a peroxidase stain. Resident rat myocardial leukocytes were primarily mast cells and macrophages; no resident polymorphonuclear leukocytes were present (Table 1). Resident leukocytes were interstitial (not intravascular) and frequently perivascular.

Peroxidase as a Histochemical Marker of Resident Rat Myocardial Mast Cells

Mast cells were identified with a myocardial peroxidase stain. The number of cells in 5 μm sections that were peroxidase positive was determined by counting cells staining positive using the dianisidine histochemical stain. In calibrated fields from sections from four hearts (100 fields per heart) an average of 0.85 peroxidase-positive cells/mm² (0.67–1.87 range) was identified. Figure 1 is a representative section from a heart demonstrating...
TABLE 1. Resident Leukocyte Population

<table>
<thead>
<tr>
<th>Subset</th>
<th>Heart 1</th>
<th>Heart 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mast cells</td>
<td>2.09/mm²</td>
<td>1.25/mm²</td>
</tr>
<tr>
<td>Macrophages</td>
<td>2.87/mm²</td>
<td>1.04/mm²</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.09/mm²</td>
<td>0.01/mm²</td>
</tr>
<tr>
<td>Platelets</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>PMNL</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>

PMNL, polymorphonuclear leukocytes.

The resident leukocyte population was characterized by immunofluorescence with antibodies directed at the indicated subsets of rat leukocytes. Two hearts perfused with normoxic perfusate for 35 minutes were studied in this fashion, a total of 100 fields per heart were examined. Values are expressed as the average number of cells identified per square millimeter in 5 μm thick sections.

Peroxidase-positive cells histologically distinct from myocytes. Peroxidase-positive cells were typically round, with single nuclei, and stained avidly positive for granular peroxidase. In addition, there was a notable absence of nonspecific myocyte peroxidase positive staining. To confirm that the cells that stained positive for peroxidase were mast cells, slides from sequentially cut frozen sections were alternatively stained for peroxidase or specific leukocyte subsets with the immunoalkaline phosphatase technique (Figure 2). With this technique, only cells identified as mast cells were found to be peroxidase positive. The percentage of mast cells staining positive for peroxidase was determined by comparison of the histochemical count of peroxidase positive cells to an independent count of the number of cardiac mast cells made using Toluidine blue stained sections. In three hearts (400 fields per heart), the density of mast cells by Toluidine blue was 1.02/mm². Thus, approximately 83% of the resident cardiac mast cells (0.85/1.02) were peroxidase positive.

Time Course of Mast Cell Degranulation During Myocardial Perfusion

No peroxidase activity was detected in the effluent of 16 hearts during either a 35 minute prehypoxic perfusion or 60 minutes of hypoxia. However, upon reoxygenation, an immediate and sustained release of peroxidase was detected from all hearts. This is demonstrated in Figure 3 where the peroxidase release for three hearts is shown over the entire perfusion period (prehypoxic, hypoxia, and reoxygenation). The normalized peroxidase release was highest just at the moment of reoxygenation.

The biochemical characteristics of the eluted peroxidase were examined. The pH optimum of the peroxidase from both effluent and heart was 5.5, typical for mast cell and leukocyte peroxidases (Figure 3 insert and Henderson and Kaliner). Peroxidase activity was also unchanged following an overnight dialysis, and it was totally inhibited by the addition of 0.2% sodium azide.

Relation of Peroxidase Release to Myocardial Injury

To study the relation between mast cell degranulation and myocardial damage during reoxygenation peak peroxidase release was compared to total creatine kinase release. The highest peroxidase concentration was always found in the coronary effluent collected between 3 and 6 minutes of reoxygenation. This value was used to indicate the extent of mast cell degranulation and was correlated to the integrated creatine kinase release over 18 minutes of reoxygenation. The correlation over a wide range of injury is shown in Figure 4. The mean total creatine kinase release was 1,285 units/g dry wt (range 606 to 2,436), and the mean peroxidase release was 384 units/min/g dry wt (range 143 to
The correlation coefficient relating creatine kinase release to peroxidase release was 0.96.

Reoxygenation Injury in the Presence of a Mast Cell Inhibitor

To study the role of the resident cardiac mast cell as a mediator of reoxygenation tissue injury the same perfusion protocol of normoxic washout followed by hypoxia and reoxygenation was repeated using six additional hearts in the presence of 10 \( \mu \)M Lodoxamide Tromethamine, a potent mast cell stabilizer. In these six hearts, peak peroxidase release was attenuated by a factor of 7.8 compared with the 16 untreated "control" hearts previously shown in Figure 4 (control, 384±174 units/min/g dry wt [mean±SD]; Lodoxamide, 49±21 units/min/g dry wt, \( p<0.001 \); Figure 5). This reduction in peroxidase release was accompanied by a marked decrease in myocardial injury as determined by total creatine kinase release (control, 1,285±500 units/g dry wt; Lodoxamide, 217±117 units/g dry wt, \( p<0.001 \)), and wet weight-to-dry weight ratio (control, 8.40±1.52; Lodoxamide, 5.71±0.38, \( p<0.001 \)). The protective effect of Lodoxamide was not related to different coronary flow rates as there was no difference found between groups during either hypoxia or reoxygenation (Figure 6).

Discussion

Summary of Findings in This Study

In this study, numerous resident cardiac leukocytes were identified in rat hearts (Figure 1). These leukocytes were found to be approximately equally distributed between mast cells and macrophages (Table 1). These leukocytes were considered resident to the heart as they persisted within the interstitial spaces of the heart despite 35 minutes of nonrecirculating perfusion. Histochemical analysis demonstrated prominent leukocyte staining for peroxidase which localized to the resident mast cell population (Figure 2).

Using an isolated perfused heart model, mast cells peroxidase was not released during either an initial normoxic perfusion period or a subsequent 60 minute hypoxic perfusion period. However, upon reoxygenation, a prompt and sustained release of peroxidase into the coronary effluent was seen, indicating mast cell degranulation (Figure 3). When the extent of myocardial damage was compared to the degree of mast cell degranulation a close linear relation was found (Figure 4). Marked reductions in myocardial injury in this model were seen when mast cell degranulation was prevented with Lodoxamide Tromethamine (Figure 5). One interpretation of these results is that the resident cardiac mast cells are a critical component of early myocardial reperfusion injury.

Resident Cardiac Mast Cells

Previous investigation has provided evidence for the existence of a resident population of cardiac mast cells in other species besides rat. Mast cells were originally identified histologically in post mortem human hearts, localized within the myocardium usually adjacent to small capillaries and venules.29 Moreover, normal human myocardial mast cell density is greater (1.8–4.0 cells/mm\(^2\)) than the density

![FIGURE 4. Peak peroxidase release (y axis) correlated closely (\( r=0.96 \)) with the extent of myocardial damage (total creatine kinase release, x axis).](image-url)

![FIGURE 5. Lodoxamide Tromethamine (U-42, 585E) at a concentration of 10 \( \mu \)M substantially reduced peroxidase release (mast cell degranulation), creatine kinase release (myocardial injury) and wet weight-to-dry weight ratio (myocardial edema) after reoxygenation (\( p<0.001 \) for all three). Hearts (n=16 untreated control, n=6 treated) were perfused for 35 minutes with normoxic nonrecirculating perfusate, 60 minutes with substrate free hypoxic perfusate, and 18 minutes with substrate free normoxic perfusate in the absence or presence of Lodoxamide (see "Materials and Methods").](image-url)
that we found in rats.\textsuperscript{39,40} The presence of mast cells may have been indirectly identified in canine hearts. In two previous studies in which myocardial peroxidase levels were measured as a marker of polymorphonuclear infiltration, baseline myocardial levels were detected with the same peroxidase assay used in this study.\textsuperscript{41,42} A baseline nonzero level suggests myocardial mast cells are resident in dog hearts. Finally, resident mast cells and histamine release during myocardial ischemia and reperfusion have been demonstrated in guinea pig hearts.\textsuperscript{43} The widespread presence of cardiac mast cells suggests the potential for mast cell–mediated reperfusion injury in other species besides rat.

Acute Myocardial Infarction and Mast Cells

Other studies have provided evidence that mast cells may potentiate tissue injury during acute myocardial infarction. Masini has recently shown that both histamine and lactate dehydrogenase release are reduced when reperfusion occurs in the presence of cimetidine or reduced glutathione after myocardial ischemia in the isolated perfused guinea pig heart.\textsuperscript{43} That study, albeit limited in scope, suggested that inhibition of mast cell degranulation with these agents resulted in enhanced myocardial salvage.

In a nonreperfused canine model of myocardial ischemia, DeBoer et al demonstrated a significant reduction in tissue damage when animals were pretreated with Lodoxamide before the onset of ischemia.\textsuperscript{44} In that study, myocardial infarct size, in the setting of similar regions at risk, was reduced 56\% in the treated group after 6 hours of coronary occlusion. The beneficial effect of this drug was attributed to stabilization of mast cells. In a similar experiment with 90 minutes of coronary occlusion followed by reperfusion, Lodoxamide reduced infarct size from 47\% of the region at risk to 21\%.\textsuperscript{45} The beneficial effect of the drug in this latter study was demonstrated not to be a function of changes in blood flow or antithrombotic effects and was thought to occur through mast cell stabilization. The results of our study are consistent with these previous findings. However, unlike past studies where indicators of mast cell degranulation were not available, in our study the marked decrease in mast cell peroxidase release in hearts treated with Lodoxamide supports the hypothesis that the beneficial effect of the drug in these past two studies was mediated through mast cell stabilization.

Although the findings in this study were interpreted as showing that Lodoxamide reduced reoxygenation injury by mast cell stabilization it is possible that the drug exerted its protective effect by inhibiting xanthine oxidase.\textsuperscript{46} This appears unlikely because the concentration of Lodoxamide used in this study (10 \(\mu\)M) is below the I\textsubscript{50} (57 \(\mu\)M) for the inhibition of xanthine oxidase. Moreover, in a recent study where Lodoxamide was used to inhibit xanthine oxidase and thereby attenuate lung reperfusion injury, millimolar concentrations of the drug were required.\textsuperscript{47}

Mechanism by Which Mast Cells Could Mediate Early Reoxygenation Injury

Rat mast cells are known to contain potent preformed chemical mediators including histamine, serotonin, proteases, superoxide dismutase, and peroxidase.\textsuperscript{36,48} In addition, mast cells synthesize significant quantities of leukotrienes when stimulated.\textsuperscript{49} The release of histamine, serotonin, and various leukotrienes might immediately effect coronary vascular resistance and permeability and precipitate reoxygenation damage by increasing tissue edema and no-reflow. In this study, there was no observable difference in coronary flow during hypoxia or reoxygenation between treated and control hearts. Since hearts were perfused at a constant pressure, the lack of differences in coronary flow suggests that the coronary vascular resistance was comparable. Thus, these results provide no evidence that changes in the vascular resistance by Lodoxamide was the mechanism responsible for the observed decrease in reoxygenation injury. Nonetheless, the difference in the wet weight-to-dry weight ratio between the two groups may indicate
that Lodoxamide preserved the permeability of the microvasculature.

Peroxidase from the mast cell may contribute directly to tissue injury. Released mast cell peroxidase has been shown to be toxic to cells in vitro through enzymatic generation of hypohalous acids in the presence of halides and hydrogen peroxide.49 Thus, one interpretation of our data is that in the perfused heart the release of mast cell peroxidase and mast cell superoxide dismutase50 in the presence of chloride and superoxide would result in the enzymatic generation of significant quantities of hypochlorous acid mediating tissue injury.49

Potential Significance of the Results of This Study

These data suggest the following: First, previous investigation of reperfusion injury in the isolated perfused heart model has been invariably interpreted using the assumption that the preparation is leukocyte free. Specifically, studies of ischemia or hypoxia in perfused hearts have either not considered the resident leukocytes9-51 or have concluded that because the preparation is perfused with a blood-free perfusate, leukocytes were not present.52-54 Data presented in this study indicate that this assumption is incorrect and that results obtained with the perfused rat heart model should be interpreted with due consideration given to the resident leukocyte population. Second, this study indicates that leukocytes normally resident to the heart may mediate tissue injury during reoxygenation independent from migrating polymorphonuclear leukocytes.9-28

Conclusions

 Resident rat myocardial leukocytes are primarily macrophages and mast cells. During reoxygenation injury mast cells release a peroxidase, and the extent of tissue injury is related to the amount of peroxidase released. When mast cells are inhibited from degranulating during reoxygenation important reductions of tissue injury are seen.

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**KEY WORDS** • heart • mast cell • reperfusion
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A M Keller, R M Clancy, M L Barr, C C Marboe and P J Cannon

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