Cryogenic Preservation of Monocytes From Human Blood and Plateletpheresis Cellular Residues

By S. M. Hunt, F. J. Lionetti, C. R. Valeri, and A. B. Callahan

A separation procedure was devised for the isolation of human monocytes from buffy coat of whole blood and the cellular residues in platelet bags after plateletpheresis of donors with the Haemonetics Model 30 Cell Processor. The mononuclear cells were isolated using Ficoll-Isopaque, and monocytes were then separated from lymphocytes by centrifugal cytography using two separation chambers in a Beckman JE-6 rotor. Approximately 30 × 10⁶ monocytes were obtained from 100 ml of whole blood. These were 93% myeloperoxidase positive (MPO), while 98% showed intact membranes in the fluorescein diacetate (FDA) ethidium bromide (EB) test. Eighty-seven percent ingested 1–5 or more opsonized latex particles, and 93% ingested 1 or more ethidium-treated zymosan particles. In three procedures, 10⁶ monocytes were obtained from cellular residues in platelet bags. Monocytes were frozen using 5% dimethylsulfoxide (DMSO), 6% hydroxyethyl starch (HES), 4% sucrose, and 56 mM glucose in Normosol-R, pH 7.1. Two-milliliter aliquots containing 6.7 × 10⁶–7.2 × 10⁶ monocytes were cooled at 4°C/min to –80°C, transferred, and stored for 1–3 mo in liquid nitrogen. A total of 25 × 10⁶–30 × 10⁶ monocytes were frozen in each of 5 separate experiments. Monocytes from platelet bags were frozen identically using 2.0-ml aliquots containing 60 × 10⁶–70 × 10⁶ cells. After storage for periods up to 3 mo, they were thawed. In some studies, the monocytes were diluted to reduce the DMSO to 1.25%, and in others the monocytes were washed with a four-fold volume of the solution used to freeze them, but without DMSO. Ninety-eight percent of monocytes frozen were recovered morphologically intact, 90% were MPO positive, and 87% were FDA positive. The diluted monocytes were incubated at 37°C for one-half hour with latex or yeast and washed twice to remove excess particles. Ninety-four percent ingested latex and 89% ingested yeast. Slightly higher fluorescence tests and similar ingestion indices were obtained with the larger numbers of monocytes derived from plateletpheresis cellular residues. The results demonstate the utility of centrifugal cytography as a means to isolate monocytes, which can be cryogenically preserved for months with small loss of function.

THE STUDY of human monocytes is encumbered because of their relatively small numbers in peripheral blood and difficulty with their isolation due primarily to their adhesiveness to wettable surfaces. The fundamental role of monocytes in the immune response to soluble and particulate antigens could be investigated more efficiently and better understood with improved methods for their isolation and long-term preservation. Principles employed for separation include gradient centrifugation on Ficoll-Isopaque, albumin, sucrose, adherence to glass or plastic, selective adsorption to nylon and centrifugal centrifugation. Preservation has been limited to brief periods of storage at 4°C, while cryogenic methods for mononuclear cells stressed the preservation of lymphocytes for antigen typing or antibody testing and did not resolve the lymphocytes from the monocytes prior to freezing.

We have employed counterflow centrifugation (elutriation) to isolate granulocytes in high yield and purity from guinea pig, baboon, and human whole blood and have preserved them using a combination of HES and DMSO. Likewise, we found that counterflow centrifugation of the buoyant fraction obtained with Ficoll-Isopaque after centrifugation of whole blood or the residual cells in platelet bags after plateletpheresis adapted easily to the separation of monocytes from the smaller lymphocytes. Pheresis procedures for the collection of platelets provide a source of large numbers (10⁶–10⁷) of mononuclear cells, which occur as contaminating cells in the platelet suspensions and are removed from the bags as by-products. Monocytes subjected to the cryogenic procedure for granulocytes, we found, could be stored for months in liquid nitrogen with small losses of cells or diminution of function. This article describes the isolation of monocytes by centrifugal cytography and their cryogenic preservation.

MATERIALS AND METHODS

Blood Collection

One hundred milliliters of whole blood was collected from healthy donors into two 50-ml plastic bottles each containing 7.2 ml of citrate-phosphate-dextrose (CPD) and aseptically transferred into 450-ml polypropylene screw cap Corning centrifuge tubes. They were centrifuged in an International centrifuge at 350 g for 28 min at room temperature (22°C). The plasma was removed aseptically, put into a separate 50-ml tube, and 4-6 ml of buffy coat and

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upper packed cell layer aspirated into separate tubes using Silicolad-treated Pasteur pipettes. The plasma was added back, the tubes mixed in a vortex mixer for 10 sec, and the mixture centrifuged a second time for 20 min at 350 g. Four milliliters of the buffy coat were removed and added to the cells from the first centrifugation. Residual blood cells in platelet bags after plateletpheresis of normal health donors with a Haemonetics Model 30 Cell Separator were obtained. The residual 10–15 ml of pelleted blood cells was the starting material for monocyte isolation.

Isolation of Monocytes From Whole Blood

Buffy coats were diluted threefold with one part autologous plasma and one part isotonic saline containing 0.01 M phosphate, pH 7.1. Thirty-five milliliters of the diluted buffy coat was layered on 15 ml of Lymphoprep (Ficoll-Isoopaque, Nyegaard & Co., A/S, Oslo, Norway, specific gravity of 1.077 g/ml), the buoyant mononuclear layer isolated, the mononuclear cell fraction pelleted, and resuspended in 20–25 ml buffered NaCl (0.15 M), pH 7.1, with glucose (0.028 M) and human albumin (1.2 g/dl).

Separation of monocytes from lymphocytes was effected by counterflow centrifugation in a Beckman J-21B centrifuge as has previously been described for the isolation of granulocytes. However, for monocytes, two separation chambers were used and a slightly hypertonic medium, resulting in less cell loss and finer control of separation. Flow rates of buffer and cells passing through the rotor were controlled by a Cole-Palmer 7545 Masterflex pump with a 10 turn external potentiometer that produced flow rates from 7 to 20 ml/min in 0.2 ml increments. The 20–25 ml of mononuclear cell suspension were mixed in an external chamber and pumped through the rotor at a flow rate of 8.2 ml/min. Monocytes exited the rotor when the flow rate was increased to 8.8 ml/min after 20 min, and to 9.4 ml/min after another 20 min for approximately 60 min. Monocytes remained in the separation chambers and pelleted when the buffer flow and centrifuge was stopped. Monocytes were aspirated into a 10-ml plastic syringe.

Monocytes From Pheresis Residues

Residues (10–15 ml) were diluted threefold with 2 vol of AB plasma and PBS buffer (1:1). The buoyant mononuclear fraction was obtained with Lymphoprep and subjected to counterflow centrifugation as with whole blood. From two chambers in the rotor, 7.0–8.0 ml of monocyte suspension was obtained, diluted 1:2 with counterflow buffer, and divided into 2-ml aliquots containing 60 × 10⁶–70 × 10⁶ monocytes.

Size Distributions

Size distributions were obtained with a Coulter Counter Z1H particle counter with Channelyzer and X-Y plotter. Monocytes were counted by integration of the cell numbers within each population. Platelet, red cell, and lymphocyte contaminations were determined in the same way on the monocyte samples in the chambers and cells in the effluent during the isolation. Cell suspensions counted were (A) 9.9 ml of buffer plus 0.1 ml of monocytes, (B) 9.8 ml of preservative solution plus 0.2 ml of prefreeze monocytes or thawed monocyte suspension, and (C) 9.2 ml of a 1:3 dilution of the freezing solution plus 0.8 ml of thawed diluent cells. Cells counts were also made with a 1:500 dilution of monocyte suspensions before and after lysis of contaminating red cells with Zap-isoton in a Coulter Counter, Model F. Red cells were counted as the difference between non-Zap-isoton treated and Zap-isoton treated aliquots.

Morphology

Cellular identifications were made from Wright's and fresh myeloperoxidase-stained smears.

The recovery of monocytes in the different preparations was derived from the total white count, the percentage of myeloperoxidase-positive cells, and the percentage of granulocytes in the differential white cell count.

Microfluorescence observations were made with an Olympus Vanox transmission fluorescence microscope. Thin wet-mounted preparations were incubated in a dye solution containing a combination of fluorescein diacetate (Sigma) (FDA) and ethidium bromide (Sigma) (EB) as described. Membrane integrity was estimated as green fluorescence in cells where FDA entered the cytoplasm and produced visible fluorescein in intact cells. The integrity of nuclear membranes was assessed as the percentage of monocytes that produced intense red nuclear fluorescence when EB, normally excluded, entered nuclei and interacted with DNA.

Particle Ingestion by Monocytes

Phagocytic properties of monocytes were measured as their ability to ingest opsonized latex and zymosan particles. Fluorescent latex (Fluolite DS-5005) was obtained from ICI, Finland and used as described. At a particle-to-cell ratio of 150:1, a dependence of ingestion on serum opsonization has been shown. Wet mounts were prepared and 200 cells counted. The percentage of cells that ingested particles was graded as: zero, no particles; low, 1–5; medium, 5–15; and high, more than 15 particles per cell.

Zymosan (Sigma, S. cerevisiae) ingestion was carried out in a similar manner. Ingestion and fluorescence of 200 cells were scored. Zymosan ingestion was rated as: zero, none; medium, those with 1–5 particles; and high, more than 5 particles. Controls with PBS substituted for serum gave little or no reaction with either zymosan or latex.

Cryopreservation of Monocytes

Monocyte suspensions isolated from whole blood or platelet bags were preserved by freezing using the procedure for granulocytes previously described. Briefly, a volume of cryoprotectant solution at 4°C equal to the volume of suspension obtained from each chamber (3.5–4.0 ml) was added dropwise with stirring. The final composition of the mixture was 5% DMSO, 6% HES, (McGaw, M.W. 150,000) 4% human serum albumin, and 56 mM glucose in Normosol-R (Abbott), pH 7.1. The mixture was divided into 2-ml aliquots in 17 × 100 mm polypropylene tubes, held at 2°C for 5–10 min, frozen at 4°C/min to −80°C for 40 min, and stored in liquid nitrogen at −197°C for an average of 37 days. Unfrozen monocytes were reserved as controls for cell counts, volume distributions, myeloperoxidase activity, microfluorescence, and particle ingestion studies.

Frozen monocytes were thawed at 49°C/min in a 42°C water bath. The tubes were shaken manually until all ice melted except for a pellet of approximately 3 mm diameter. The tubes were removed and swirled until the pellet melted, at which time the thawed samples were cool to the touch (8°C–10°C). They were then maintained at 2°C in ice for washing and testing.

Thawed monocytes were diluted 1:4 dropwise (1 drop/4–5 sec for 3 min) with 3 vol of a solution at room temperature composed of 6% HES, 4% human serum albumin (HSA), and 28 mM glucose in Normosol-R, pH 7.1. The thawed diluted suspension was assayed for counts, size distribution, microfluorescence, myeloperoxidase activity, and particle ingestion as described above.

RESULTS

Volume distributions of mononuclear cells from whole blood subjected to incremental flow–counterflow centrifugation are shown in Fig. 1. The first
Fig. 1. The isolation of monocytes from the buoyant mononuclear cell layer of whole blood isolated with Ficoll-Isopaque. Volume plots of the number and types of cells at various flow rates of buffer through the rotor are shown. The numbers in parenthesis below each flow rate are the times in the separation procedure (in minutes) at which the samples were taken. Numbers above the peaks are median channel numbers (midranges). The numbers under the peaks are the cell numbers (in thousands) within the distribution. P + R are platelets plus red cells, and the areas delineated by L are lymphocytes. The final plot shows the distribution of cells left in the chamber. The shaded area is monocytes.

distribution at time zero (marked Lymphoprep) defines the composition of the sample obtained with Lymphoprep. Platelets (P, median channel 4, plus red cells, R), lymphocytes (L, median channel 34), and monocytes (M, median channel 69, the shaded area in Fig. 1) were the major components. At 8.2, 8.8, and 9.4 ml/min, platelets, red cells, and lymphocytes exited, accompanied by small numbers of monocytes. After 60 min at 9.4 ml/min, the first chamber in the rotor contained 89% of available monocytes in the Ficoll-Isopaque buoyant cell layer. None were found in the second chamber under these conditions. However, its use permitted recovery of monocytes that spilled over from chamber I when large numbers in plateletpheresis cell residues were processed. The volume spectrum was one primarily of monocytes (shaded area, median channel 72). The small peak at channel 7 consisted of cell fragments and red cells, while the shoulder to the left of the monocyte distribution consisted of large lymphocytes.

Monocytes were isolated in five experiments with whole blood. Of $127 \pm 4.0 \times 10^6$ (average \pm SE) monocytes available in 100 ml of whole blood, $69 \pm 12 \times 10^6$ (65%) were recovered from buffy coat, $33 \pm 16 \times 10^6$ (26%) were recovered with Ficoll-Isopaque, and $25 \pm 9.0 \times 10^6$ (20%) recovered in the chamber after incremental flow centrifugation. The greatest losses of monocytes occurred in preparing buffy coats and buoyant monolayer, while only a small loss of monocytes occurred in the JE-6 rotor. Wright's stained smears showed isolated monocytes to be 99% mononuclear. The midrange of the volume distribution (71) was consistent with a population of large mononuclear cells. Contamination with PMNs or other cells was less than 1%. All of the cells (98%) produced fluorescein in cytoplasm from fluorescein diacetate and few were ethidium positive (2%), indicating intact cytoplasmic and nuclear membranes. Ninety-three percent were myeloperoxidase positive, while the median peak channel (71 \pm 3) was unchanged, showing that cell volumes remained constant during the isolation. Nearly all were capable of ingesting oponized latex and zymosan (see Table 1, control).

Monocytes isolated from platelet bags after pheresis of 3 donors were present in mixed leukocytes, which ranged from $2.0 \times 10^7$ to $7.5 \times 10^7$. Of $4.3 \pm 1.7 \times 10^9$
leukocytes available in the average concentrate, 94% were mononuclear, 5% were granulocytes, and 28% were myeloperoxidase positive, indicating \(1.1 \times 10^9\) of the total were monocytes. Recoveries of monocytes at these large magnitudes were much higher than from whole blood. Ninety-one percent of monocytes were recovered after centrifugation with Ficoll-Isopaque, while 28% were granulocytes, and 2% were lymphocytes. Nearly all (97%) of monocytes were recovered after being frozen, thawed, diluted, incubated, and washed. These remained morphologically intact without swelling or shrinking. In contrast, of monocytes frozen similarly without cryoprotectant (Fig. 2, insert), very few cells were “viable” in the fluorescein diacetate test. This was also reflected by the median channel numbers, which did not vary, showing maintainance of volume during the separation.

Preservation of monocytes with HES and DMSO in hyperosmolar media was undertaken with the same protocol previously employed for human, baboon, and guinea pig granulocytes. Volume plots of monocytes as collected, mixed with cryoprotective media, frozen, thawed, diluted to reduce DMSO, and incubated with particles to assess ingestion, revealed monocytes to be remarkably stable (Fig. 2). The constancy of the shape of the volume plots during the steps of freezing, thawing, dilution of DMSO, and incubation with particles gives evidence that monocyte volume was well maintained during the osmotic stresses incurred in the various steps in the procedure. The volumes were made to be smaller than normal with hyperosmolal buffer (300 mmole/kg H2O) monitored by median channel numbers. The values of 59 for thawed, 59 for thawed and diluted, and 62 for the incubated cells close to 61, the value for monocytes as isolated. The number of cells within the monocyte distribution indicated that 97% of monocytes separated were recovered after being frozen, thawed, diluted, incubated, and washed. These remained morphologically intact without swelling or shrinking. In contrast, of monocytes frozen similarly without cryoprotectant (Fig. 2, insert), very few cells survived freezing and thawing. This is evident from the absence of a cell population at channel 60, the presence of a large population of cell fragments at channel 20, and the inability to detect intact monocytes in stained smears.

Recoveries of monocytes preserved by freezing estimated from volume plots, white cell numbers, differential counts, and myeloperoxidase-positive cells are summarized in Table 1. Of \(25 \pm 9 \times 10^6\) total monocytes in 4 tubes, 100% were recovered after freeze preservation. Therefore, the only significant loss of cells occurred during their isolation from whole blood, i.e., the preparation of buffy coat and mononuclear cell fraction.

The stability of monocyte membranes determined by fluorescein production from FDA and exclusion of

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Monocytes (\times 10^6) (n - 5)</th>
<th>Recovery (%)</th>
<th>Myeloperoxidase Positive (%)</th>
<th>Microfluorescence (%)</th>
<th>Ingestion (Percent of 200 Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-frozen</td>
<td>25 - 11 (16-37)</td>
<td>98 ± 5</td>
<td>(89 \pm 11)</td>
<td>96 ± 2.0</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Thawed</td>
<td>25 - 10 (17-39)</td>
<td>98 ± 4</td>
<td>(89 \pm 10)</td>
<td>96 ± 2.0</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>Thawed and diluted</td>
<td>25 - 10 (15-40)</td>
<td>99 ± 1</td>
<td>94 ± 5</td>
<td>95 ± 2.0</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Thawed dilute incubated washed*</td>
<td>24 - 10 (93-100)</td>
<td>98 ± 4</td>
<td>(90 \pm 11)</td>
<td>87 ± 5.0</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>Control</td>
<td>25 - 9.0 (16-29)</td>
<td>93 ± 5</td>
<td>98 ± 1.0</td>
<td>2 ± 1</td>
<td>37 ± 18</td>
</tr>
</tbody>
</table>

The mean ± standard error and the range (parentheses) is given. Recovery was calculated from counts with the Counter, Counter Model F (upper number) also from counts of cells within the monocyte population obtained with the Coulter ZH Channelizer (lower number). Recoveries are based on the prefrozen value as 100%. The recovery prefrozen is the percentage of monocytes isolated.

*Particles were incubated with the thawed, diluted suspensions at 37°C for 30 min and the cells washed twice to remove excess particles. Other tests were made on unwashed suspensions.

Latex phagocytosis: high, more than 15; med, 5-15; low, 1-5; and zero, no particles/cell. Yeast phagocytosis: high, more than 5; low, 1-5; zero, no particles/cell.

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EB from nuclei, as well as phagocytic functions also manifested the stability of monocytes after freeze preservation (Table I). Comparison of the control cells, those mixed with cryoprotectants (prefrozen), and thawed cells revealed small changes in cell numbers, FDA, and EB reactivity. Thawed monocytes, incubated with particles and washed, incurred no loss in cell numbers, but a decrease in cytoplasmic fluorescence (FDA) to 87% and an increase in nuclear permeability to EB to 13%. Of the incubated twice-washed monocytes, 94% ingested opsonized latex (50% ± 21% high, 35% ± 12% medium, 9% ± 9% low). These magnitudes were similar to those of the unfrozen controls (37% ± 18% high, 39% ± 20% medium, and 9% ± 7% low). Likewise 89% of opsonized ethidium-treated zymosan (yeast) was ingested by frozen–thawed monocytes (31% ± 21% high, 58% ± 21% low) compared to 94% for fresh monocytes (64% ± 15% high, 30% ± 15% low). The major difference due to freezing was observed with yeasts where the high capacity for ingestion of yeast dropped from 64% high in fresh to 31% in thawed cells, indicative of a reduction in avidity for particles due to freezing. Phagocytosis could not be measured in DMSO at the 5% concentration. As reported for granulocytes, myeloperoxidase was also inhibited.

Results of freezing experiments with monocytes from plateletpheresis cellular residues were similar to those obtained with whole blood. Because of the greater numbers of monocytes available (1 × 10⁸–5 × 10⁸), they were divided into aliquots containing 50 × 10⁶ monocytes in order to be frozen with the same method. The contents of both chambers of the rotor were preserved in an identical manner with similar results. The recoveries of monocytes at each stage showed small losses occurring after thawing and diluting (to reduce the DMSO concentration) and after incubation with particles and subsequent washing. In vitro function tests of thawed monocytes from pheresis cell residues were routinely in the 90th percentile range in all tests (not shown), offering evidence that human mononuclear cells are strongly resistant to cryoinjury to which polymorphonuclear cells are susceptible.
DISCUSSION

Studies of monocyte physiology have been limited by the relatively small numbers that occur, that can be isolated practically from blood, and their innate adhesiveness, which has made it difficult to isolate them in suspension. Gradient isolated monocytes are often few in number and have variable purity, while the Ficoll-Hypaque-treated adherent cells are purer, but difficult to detach from the adhering surface without damage or altered function. The counterflow centrifugation elutriation method, on the other hand, isolated monocytes in suspension, as described by Sanderson et al.9 This utilized a single chamber in which 1.5 x 10^6–3.0 x 10^6 monocytes of 90% purity and 99% functionality were isolated from 10 ml of whole blood. The methods employed in this paper were similar except for minor modifications. A second separation chamber was added for use with larger volumes of buffy coat and higher numbers of monocytes. Minor changes in g-forces and buffer flow rates were used to effect the separation. Major advantages of counterflow centrifugation were that mononuclear cells were separated on the basis of size at low net g-force and the monocytes were in suspension, minimizing adhesion forces frequently used to achieve their isolation.

Monocytes isolated from whole blood or plateletpheresis cellular residues using the centrifugal counterflow elutriation principle were found to have a high proportion with intact cytoplasmic and nuclear membranes as tested by fluorescein diacetate and ethidium bromide, respectively. There was also high reactivity of myeloperoxidase, and ingestion assays revealed high values for ingestion of yeast and latex.

Dean and Strong10 demonstrated the feasibility of cryopreservation of mononuclear phagocytes with 7.5% dimethylsulfoxide in 10% heat-inactivated fetal bovine serum. However, the population frozen was approximately 85% lymphocytes and only 15% (1 x 10^9–2 x 10^9) monocytes. In our studies with relatively pure monocytes, we employed a lessor concentration of DMSO combined with HES, causing less cytotoxicity and effecting better preservation. We showed previously with granulocytes that DMSO between 5% and 10% caused swelling, dependent on concentration and temperature.19 This was accompanied by losses of membrane integrity, and cytoplasmic fluorescein, an increase in nuclear fluorescence with ethidium, and a cessation of particle ingestion. Additionally, DMSO in excess of 1.25% inhibited granulocytic phagocytosis and myeloperoxidase.18

In this study with monocytes, minimization of swelling was facilitated by monitoring volume (size) changes of the cells. Sizing was done before freezing to determine rates of admixture with cryoprotective solutions and after thawing to effect washing with minimal volume changes. Use of hyperosmolar media maintained the cells at smaller volumes thereby reducing swelling effects of DMSO, and the ensuing cellular damage and loss of viability after freezing. By combining HES and DMSO, effective cryopreservation was achieved at lower DMSO concentrations (5%) without permitting monocytes to exceed initial volumes as collected, preventing the deleterious effects of swelling. Although high recoveries and function of monocytes after freezing reflect an inherent stability greater than that of granulocytes, use of principles and procedures developed for granulocyte isolation and cryopreservation are in part responsible for the results described here. These emphasize hyperosmolar media, combined cryoprotectants HES and DMSO, and concentrations of DMSO adequate for cryopreservation without membrane disruption and loss of function.

In our approach it is highly advantageous that plateletpheresis as performed with the Haemonetics Model-30 processor concentrates billions of monocytes in the cellular residues. Using this starting material, it was possible to isolate 1.0 x 10^9 mononuclear leukocytes that were 95% myeloperoxidase positive consisting of 94%–95% monocytes. This is 20 times that which we isolated from 100 ml of whole blood and 200–400 times the number of monocytes isolated by Sanderson et al.9 from 10 ml of whole blood.

The availability of a large number of homogeneous monocytes renders their preservation desirable, primarily for future use or study. Fortunately, this can be accomplished with existing technology for preserving granulocytes.4,6 Monocytes from one donor can be isolated, divided into aliquots, preserved and used for analytical purposes or cell studies. The opportunity to perform monocyte transfusions when indicated may now be contemplated. Studies are now in progress that seek to evaluate in vivo in baboons the therapeutic effectiveness of cryopreserved monocytes.

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Cryogenic preservation of monocytes from human blood and plateletpheresis cellular residues

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