THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 265, No. 5, Issue of February 15, pp. 2584-2587, 1990
Printed in U.S.A.

Millimolar Concentrations of Ascorbic Acid in Purified Human Mononuclear Leukocytes

DEPLETION AND REACCUMULATION*

(Received for publication, July 5, 1989)

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Ascorbic acid (vitamin C) was found in isolated human mononuclear leukocytes and their purified components in millimolar concentration. Intracellular ascorbic acid was depleted >96% during cell culture and was rapidly reaccumulated after addition of physiologic concentrations of ascorbic acid to the extracellular medium. Purified cells maintained concentration gradients of ascorbic acid as large as 100-fold across the plasma membrane. The ability to vary intracellular ascorbic acid concentrations over such a wide range makes it possible for the first time in these cells to study ascorbic acid function in direct relationship to intracellular concentration.

Ascorbic acid is found in human peripheral mononuclear leukocytes (1-7), yet its function and content in purified cells are unknown for several reasons. First, insensitive assay techniques have hampered measurement of ascorbic acid, which oxidizes unless strict precautions are taken (8-11). Second, the issues of ascorbic acid content, function, and transport have been confounded by uptake experiments in leukocytes using extracellular millimolar concentrations of ascorbic acid and dehydroascorbic acid (12-14). These experiments suggested that dehydroascorbic acid was transported, but ascorbic acid was not. However, dehydroascorbic acid may not be found at all in normal human plasma or at concentrations <2 μM (15), and ascorbic acid can be found not in millimolar concentration but between 50 and 150 μM (16-20). Third, it is not known if all or only some mononuclear cell types contain ascorbic acid, since highly purified cell preparations have not been studied. Finally, the fate of intracellular ascorbic acid in cultured mononuclear leukocytes and separated cell types is unknown.

Ascorbic acid function in mononuclear leukocytes can be addressed only if three prerequisites are satisfied: ascorbic acid must be measured sensitively and specifically; the vitamin concentration for each purified cell type must be determined at the time of isolation; and there must be a means to vary intracellular vitamin concentrations over a wide range for each cell type. Once ascorbic acid concentrations can be depleted and repleted for each cell class, function can be studied in direct relationship to intracellular and extracellular vitamin concentrations (21-23).

We have developed a novel high performance liquid chromatography coulometric electrochemical detection assay for ascorbic acid which has a detection limit of <1 pmol/sample and is compatible with conditions which stabilize ascorbic acid (11). We used this assay to quantitate both intra- and extracellular ascorbic acid as a function of time in cultured cells using human mononuclear leukocytes and purified B lymphocytes, T lymphocytes, and monocytes.

MATERIALS AND METHODS AND RESULTS[1]

DISCUSSION

The data indicate that purified human monocytes, B lymphocytes, and T lymphocytes each contain millimolar concentrations of ascorbic acid which are depleted during cell culture, and that reaccumulation to the initial or even higher concentrations occurs when ascorbic acid is added to the medium in physiological amounts. Depletion and reaccumulation of ascorbic acid in B lymphocytes, T lymphocytes, and monocytes provide conditions where the intracellular concentration can vary by as much as 100-fold. Using these conditions, the function of intracellular vitamin C can now be addressed directly in relationship to its intracellular concentration (21-23).

What functions might vitamin C have in these different cell types? In monocytes, it is possible that ascorbic acid is necessary to either quench or to propagate oxidants generated during the respiratory burst. These functions have been proposed for ascorbic acid in human neutrophils (31-33), another cell in which free radicals are generated during the respiratory burst (34). In B and T lymphocytes ascorbic acid function could be coupled to recognition, processing, or killing of infectious agents by these cells, linked to biosynthesis or processing of lymphokines, related to protection against oxidants, or relevant to development of drugs against infectious agents. In all 3 cell types it is possible that ascorbic acid is important in the regulation of endogenous cholesterol synthe-

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§ Supported in part by United States Public Health Service International Fellowship 1F05TW04149, the Swedish Medical Research Council, the Odd Fellows of Sweden, and the Henning and Johan Throne-Holts Foundation.

¶ Supported in part by G. E. O. Phoenix Laboratories.

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[1] Portions of this paper (including "Materials and Methods," "Results," and Figs. 1-4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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sis, since 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in these cells is inhibited by ascorbic acid (35). It is also possible that lymphocytes or monocytes contain another ascorbic acid-dependent enzyme (36).

The function of ascorbic acid in B lymphocytes, T lymphocytes, and monocytes may be meaningful in a more general sense. The presence of millimolar concentrations of vitamin C in human mononuclear cells suggests that the vitamin may play a significant role in normal cell function. Moreover, many immunological assays which use cultured lymphocytes, monocytes, or cell lines are being performed with scorbutic cells. This is due to the rapid depletion of ascorbic acid in cultured cells, and also to the lack of ascorbic acid in sera and most commercial media as a consequence of instability of the vitamin (28–30, 37). Thus, recognition of the millimolar concentrations of vitamin C in lymphocytes and monocytes *in situ* has potential application to a broad spectrum of immunological investigation.

Finally, the ability to regulate intracellular concentrations of vitamin C in immune cells is relevant to vitamin C requirements in humans. Data concerning specific biochemical reactions in direct relationship to vitamin concentration *in situ* may have application to determining vitamin requirements; the concept has been termed *in situ* kinetics (21–23). *In situ* kinetics ideally is determined in human cells where the concentration of vitamin can be exquisitely regulated, and the function of the vitamin is known (21, 22). Understanding vitamin C function in monocytes and lymphocytes is a prerequisite for determining *in situ* kinetics in these cells. By using the paradigm of repletion and depletion as shown here, these experiments in lymphocytes and monocytes are now possible.

Acknowledgments—We would like to thank Charles Carter for preparing the monocytes.

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SUPPLEMENTARY MATERIAL
TO
MILLIMOLAR CONCENTRATIONS OF ASCORBIC ACID IN PURIFIED HUMAN MONONUCLEAR LEUKOCYTES: DEPLETION AND REACCUMULATION

by
Peter Bergsten, Gabriel Amitai, John Kehrl, Kuldeep R. Dhariwal, Harvey G. Klein, and Mark Levine

MATERIALS AND METHODS

Cell isolation, purification, and culture

Mononuclear cells were prepared from normal human whole blood using Ficoll 400 and Hypaque (Histo-paque) for density gradient centrifugation (24). After isolation, cells were cultured in RPMI 1640 in the presence or absence of ascorbic acid. T lymphocytes were prepared from human tonsils by rosetting with aminoethylisothiouronium bromide treated sheep red blood cells and passage of rosette positive cells over nylon wool columns. Greater than 95% of these cells were OKT 3 positive by fluorescent activated cell sorting analysis (25). Purified T cells were cultured in RPMI 1640 with 10% fetal calf serum. B lymphocytes were prepared from human tonsils by rosetting with aminoethylisothiouronium treated sheep red blood cells, and cultured in RPMI 1640 with 10% fetal calf serum. Greater than 95% of these cells were surface immunoglobulin positive by fluorescent activated cell sorting analysis (25). Monocytes were prepared by centrifugal countercurrent elutriation of mononuclear leukocyte concentrates obtained from normal human whole blood. Monocyte purity was > 90% by histochemical staining and cell sizing (26). Monocytes were cultured in IMDM with 10% human serum.

Ascorbic acid analysis

Ascorbic acid content of all samples was measured by high performance liquid chromatography with coulometric electrochemical detection as recently described (11), with minor modifications. Ice cold solutions of either 90% methanol saturated with EDTA or 60% methanol/1mM EDTA were used as the extraction/storage solvents instead of 10% trichloroacetic acid/10 mM oxalic acid. The methanol/EDTA did not interfere with the chromatography and ether extraction was omitted. Solutions of methanol/EDTA stabilized frozen ascorbic acid samples for at least 4 weeks (unpublished observations). The column was an Ultrasphere ODS 5 um column 4.6 mm x 25 cm with titanium frits (Beckman Instruments, Altex Division).

Data analysis

Intracellular volume was estimated by an electronic particle volume analyzer (Coulter Instruments). Mixed mononuclear cells were estimated to contain 0.25 ul/10^6 cells, lymphocyte volume was 0.2 ul/10^6 cells, and monocyte volume was 0.3 ul/10^6 cells. All cell volume calculations are in agreement with previous observations (3,26,27).

Experimental points represent the mean ± S.D. of at least 3 samples. Error bars have been omitted when the standard deviation was less than the size of the symbol.

RESULTS

Ascorbic acid in mixed mononuclear leukocytes

Since ascorbic acid is unstable in biological systems, and mononuclear cells may contain ascorbic acid, we anticipated that intracellular ascorbic acid content would decrease over time during cell culture and might be restored by providing physiologic concentrations of exogenous vitamin (28,29,30). Therefore, we measured ascorbic acid content of freshly isolated mononuclear cells and cultured cells. Freshly isolated mononuclear cells contained approximately 6 mM ascorbic acid (figure 1A). We next determined whether the ascorbic acid concentration in these cells varied during cell culture. Mononuclear leukocytes were cultured either in control medium or in medium supplemented with 150 uM ascorbic acid. After twenty four hours in culture, 150 uM ascorbic acid was added to both media to determine whether cells were still capable of concentrating the vitamin. As shown in figure 1A, in the absence of ascorbic acid the mononuclear leukocyte ascorbic acid content fell from > 6mM to < 2 mM by 24 hours. When ascorbic acid was added to the control medium, the cells rapidly accumulated ascorbic acid to approximately 4 mM. In mononuclear leukocytes cultured in the presence of 150 uM ascorbic acid from the time of isolation, ascorbic acid only gradually declined to approximately 4.6 mM at 24 hours. The original intracellular ascorbic acid content was restored by addition of ascorbic acid to the medium. The gradual decrease in intracellular ascorbic acid in the supplemented cells could be explained by oxidation of ascorbic acid in the culture media, with less available for accumulation. As seen in figure 1B, ascorbic acid in medium had a half-life of approximately 6-7 hours. Less than 100 nM ascorbic acid could be detected in medium that had not been supplemented (figure 1B). Both supplemented and unsupplemented cells remained viable, as they were able to concentrate ascorbic acid against a concentration gradient after 24 hours (figure 1A). These data indicate that mononuclear leukocytes contain mM concentrations of ascorbic acid, that the intracellular concentration can be depleted during cell culture, that the concentration can be maintained or restored by addition of ascorbic acid to media, and that cells can maintain intracellular ascorbic acid as much as 50 fold higher than the extracellular milieu for at least 24 hours.

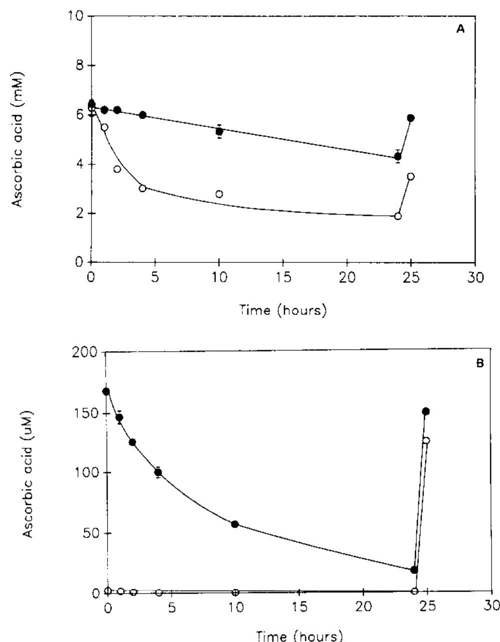

Figure 1A. Intracellular ascorbic acid concentration in mononuclear leukocytes. Freshly isolated mononuclear cells were cultured in RPMI 1640 in the presence (●) or absence (o) of 150 uM ascorbic acid. Ascorbic acid 150 uM was added at 24 hours to both groups. Ascorbic acid was measured by HPLC with coulometric electrochemical detection (see methods).

Figure 1B. Extracellular concentrations of ascorbic acid. Ascorbic acid concentration was determined in culture media of mononuclear cells in figure 1A. Media was supplemented (●) or unsupplemented (o) with 150 uM ascorbic acid at the beginning of the experiment. Both media were supplemented with 150 uM ascorbic acid at 24 hours.

Ascorbic acid in purified T lymphocytes

Since ascorbic acid is found in mononuclear cells, and mononuclear cells consist of B lymphocytes, T lymphocytes, and monocytes, we measured ascorbic acid in purified cell populations. Ascorbic acid content was measured immediately after isolation of each cell type, and also after culturing for as long as 100 hours using supplemented cells, unsupplemented cells, and cells which were first depleted and then supplemented.

T lymphocytes contained 1.0 mM ascorbic acid at the time of isolation (figure 2A). Intracellular ascorbic acid declined to <90 uM in T lymphocytes cultured for approximately 100 hours. However, when these lymphocytes were exposed to physiologic concentrations of ascorbic acid (100 uM addition), intracellular ascorbic acid accumulation was as much as 2.5 mM (figure 2A). A stepped pattern of accumulation occurred, and was due to sequential addition of 100 uM ascorbic acid and concurrent oxidation of ascorbic acid in the culture medium (figure 2C). As ascorbic acid decreased in the medium, less was available for accumulation, and accumulation plateaued or even decreased. When further ascorbic acid was added to the medium, more accumulation occurred (figures 2A, 2C). Although intracellular ascorbic acid depletion occurred in T lymphocytes in the absence of extracellular ascorbic acid, cells maintained internal ascorbic acid when 100 uM ascorbic acid was added every 12 hours to the medium (figure 2B). Thus, T lymphocytes can be depleted or repleted of intracellular ascorbic acid by the absence or presence of exogenous vitamin, and T lymphocytes supplemented with ascorbic acid concentrate as much as 30 fold more ascorbic acid than unsupplemented cells.

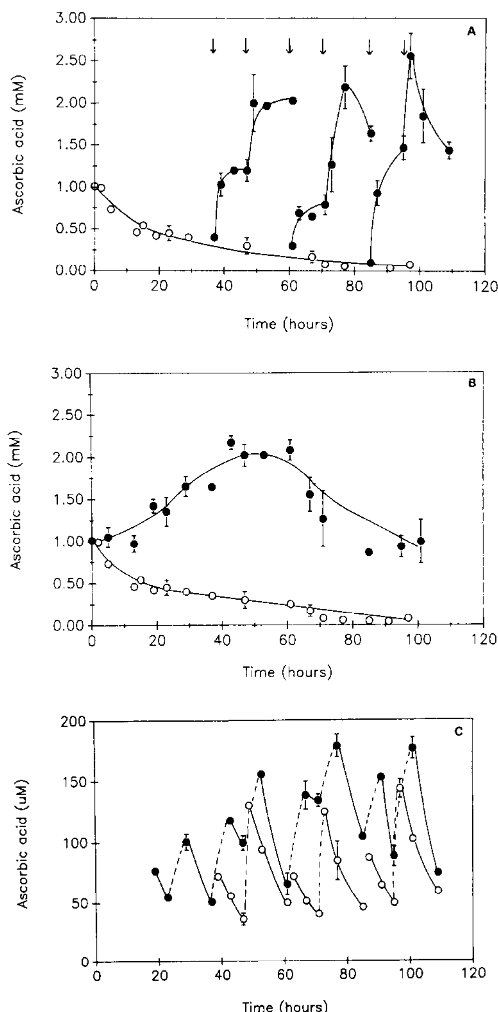

Figure 2A. Intracellular concentration of ascorbic acid in T lymphocytes. Purified T lymphocytes were cultured in RPMI 1640 with 10% fetal calf serum. Ascorbic acid (100 uM) was added to three different groups of cells grown for 38, 60, and 85 hours without ascorbic acid. Ascorbic acid additions to each group were made twice in 24 hours, as indicated by arrows. (●) represent supplemented cells; (o) represent unsupplemented cells.

Figure 2B. Intracellular concentration of ascorbic acid in T lymphocytes. T lymphocytes were cultured in the presence or absence of ascorbic acid. Ascorbic acid 100 uM was added approximately every 12 hours to supplemented cells. (●) indicate supplemented cells; (o) indicate unsupplemented cells.

Figure 2C. Extracellular concentration of ascorbic acid. Ascorbic acid concentrations in T lymphocyte culture media from figures 2A and 2B were determined before and after additions of 100 uM ascorbic acid. Ascorbic acid was measured in the media of cells supplemented approximately every 12 hours (●). Also, three separate groups of cells were depleted for approximately 38, 60, or 85 hours in a medium devoid of ascorbic acid. Beginning at each of these times, ascorbic acid was added twice during 24 hours and the media assayed for ascorbic acid (o). Dotted lines indicate that ascorbic acid was added during the interval between the two points. Cells corresponding to media (●) are shown in figure 2B and cells corresponding to media (o) are shown in figure 2A.

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Ascorbic acid in purified B lymphocytes

We performed similar experiments with B lymphocytes and monocytes, and the findings for both cell types were comparable to the T lymphocyte findings. B lymphocytes at the time of isolation contained between 1.5-3.5 mM ascorbic acid, which was depleted to < 90 uM after 80 hours in culture (figure 3A). The original internal concentration was restored or surpassed by addition of 100 uM ascorbic acid to the medium twice in 24 hours (figure 3A). As for T lymphocytes, a stepped pattern of accumulation occurred, which was due first to oxidation of ascorbic acid in the media and then to further addition of ascorbic acid to correct for oxidation (data not shown). When B cells were cultured with 100 uM ascorbic acid added every 12 hours, they maintained their internal concentration of 3 mM for $\geq$ 80 hours (figure 3B). These B lymphocytes supplemented continuously with ascorbic acid had as much as 40 fold more ascorbic acid than control cells cultured during the same time without ascorbic acid.

Ascorbic acid in purified monocytes

Monocytes also contained ascorbic acid, at a concentration of > 3 mM upon isolation (figure 4A). Ascorbic acid in monocytes was depleted to < 100 uM after 80 hours in culture (figure 4A). Similar to the findings for B and T lymphocytes, monocytes supplemented twice in 24 hours with 100 uM ascorbic acid reaccumulated the vitamin to concentrations as high as 10 mM (figure 4A). As also seen for lymphocytes, a stepped pattern of accumulation occurred, which was due first to oxidation of ascorbic acid and then to addition of fresh ascorbic acid (data not shown). Furthermore, monocytes cultured with 100 uM ascorbic acid added to medium every 12 hours also accumulated as much as 10 mM ascorbic acid (figure 4B).

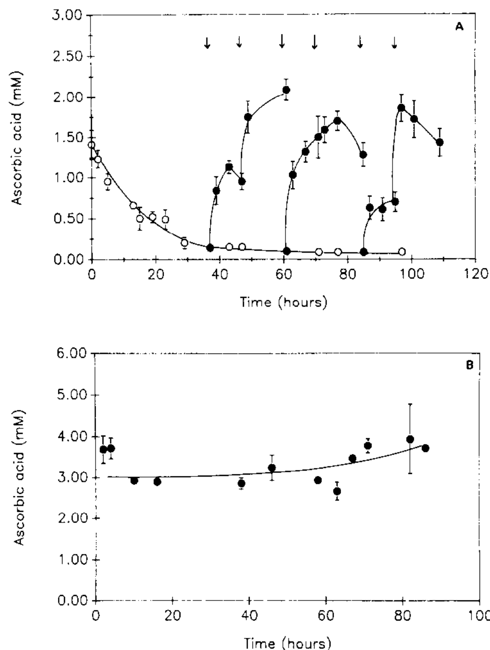

Figure 3A. Intracellular concentration of ascorbic acid in B lymphocytes. B lymphocytes were cultured in RPMI 1640 plus 10% fetal calf serum. Ascorbic acid (100 uM) was added to three different groups of cells grown for 38, 60, and 85 hours without ascorbic acid. Ascorbic acid additions to each group were made twice in 24 hours, as indicated by arrows. (●) represent supplemented cells; (o) represent unsupplemented cells.

Figure 3B. Intracellular concentration of ascorbic acid in B lymphocytes. B lymphocytes were cultured in the presence of 100 uM ascorbic acid added approximately every 12 hours.

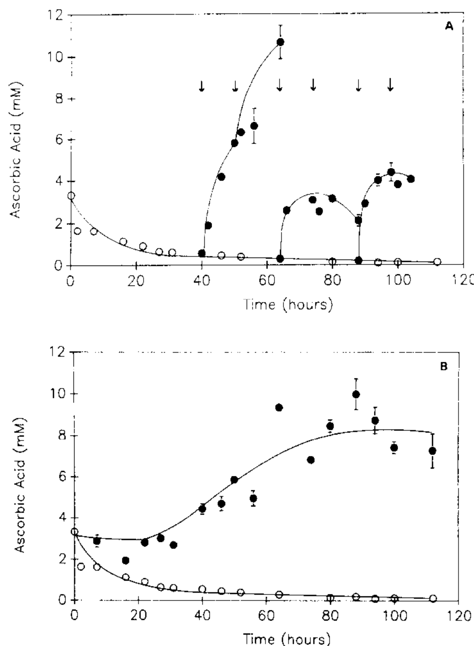

Figure 4A. Intracellular concentration of ascorbic acid in monocytes. Monocytes were cultured in IMDM containing 10% human serum (26). Ascorbic acid (100 uM) was added at the arrows to three different groups of cells. (●) indicate supplemented cells, and (o) indicate unsupplemented cells.

Figure 4B. Intracellular concentration of ascorbic acid in monocytes. Monocytes were cultured in the presence (●) or absence (o) of 100 uM ascorbic acid. Ascorbic acid 100 uM was added approximately every 12 hours to the supplemented cells.

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J. Biol. Chem. 1990, 265:2584-2587.

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