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Intra-operative preparation of autologous bone marrow-derived CD34-enriched cellular products for cardiac therapy

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Abstract

Background and Aims—With the advent of regenerative therapy, there is renewed interest in the use of bone marrow as a source of adult stem and progenitor cells, including cell subsets prepared by immunomagnetic selection. Cell selection must be rapid, efficient and performed according to current good manufacturing practices. In this report we present a methodology for intra-operative preparation of CD34⁺ selected autologous bone marrow for autologous use in patients receiving coronary artery bypass grafts or left ventricular assist devices.

Methods and Results—We developed a rapid erythrocyte depletion method using hydroxyethyl starch and low-speed centrifugation to prepare large-scale (mean 359 mL) bone marrow aspirates for separation on a Baxter Isolex 300i immunomagnetic cell separation device. CD34 recovery after erythrocyte depletion was $68.3 \pm 20.2\%$, with an average depletion of $91.2 \pm 2.8\%$ and an average CD34 content of $0.58 \pm 0.27\%$. After separation, CD34 purity was $64.1 \pm 17.2\%$, with $44.3 \pm 26.1\%$ recovery and an average dose of $5.0 \pm 2.7 \times 10^6$ CD34⁺ cells/product. In uncomplicated cases CD34-enriched cellular products could be accessioned, prepared, tested for release and administered within 6 h. Further analysis of CD34⁺ bone marrow cells revealed a significant proportion of CD45⁺ CD34⁺ cells.

Conclusions—Intra-operative immunomagnetic separation of CD34-enriched bone marrow is feasible using rapid low-speed Hetastarch sedimentation for erythrocyte depletion. The resulting

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Supplementary material available online

Procedure for red cell depletion using centrifugation of Hetastarch to be found online at: <http://www.informahealthcare.com/cyt/10.3109.14653249.2010.529888>

CD34-enriched product contains CD45⁺ cells that may represent non-hematopoietic or very early hematopoietic stem cells that participate in tissue regeneration.

Keywords

Baxter Isolex; bone marrow; CD34 selection; flow cytometry; Hetastarch

Introduction

Depending on the specific application, therapeutic cells can often be cryopreserved and stored for autologous use at a later time, or stored as cell banks for use in multiple patients. Because the product is not administered on the same day that it is manufactured, minimizing the time required for cell processing, while important for efficiency, is not critical to patient safety. In cases where the patient must be anesthetized during harvesting and administration of the therapeutic cells, it is advantageous to perform cell harvest, processing and infusion as a single procedure. This requires rapid intra-operative cell processing. In this report we describe our experience in the intra-operative preparation of CD34-enriched autologous bone marrow for cardiac therapy utilizing a rapid Hetastarch red blood cell depletion protocol followed by immunomagnetic separation of CD34⁺ cells using the Baxter Isolex 300i (Deerfield, IL, USA).

Methods

Patients

Patients ranged in age from 49 to 69 (= 60.9) years and were undergoing either coronary artery bypass graft surgery ($n = 7$) or ventricular assist device placement ($n = 3$). Patients were treated under a University of Pittsburgh (Pittsburgh, PA, USA) Institutional Review Board (IRB)-approved protocol. Products were manufactured under current Good Manufacturing Practices (cGMP) conditions at the University of Pittsburgh Cancer Institute Hematopoietic Stem Cell Laboratory under BB-IND 12304 issued by the US Food and Drug Administration.

Autologous bone marrow for therapeutic products

Heparinized bone marrow was harvested under general anesthesia from posterior iliac crests by the surgical team and was immediately heparinized. An individual experienced in bone marrow harvesting was present for the initial procedures. A pre-determined goal of 500 mL aspirated bone marrow was set, based on the anticipated maximal red cell load that could be accommodated by the Isolex following Hetastarch erythrocyte depletion. The actual volume harvested was at the discretion of the surgical team and ranged from 192 to 501 mL. Because placement of ventricular assist devices is time consuming, there was ample time for intra-operative preparation of the cellular product. Anesthesia was carefully managed for Cardiac Artery Bypass Graft (CABG) patients, for whom the graft procedure required less time than product preparation (Table I). The surgical team was notified when the product was within 1 h of anticipated delivery.

Rapid Hetastarch procedure

After accessioning the specimens and obtaining samples for flow cytometry, hematology analysis, sterility and endotoxin testing, the bone marrow was sedimented using hydroxyethyl starch (Hetastarch) for erythrocyte reduction (a detailed standard operating procedure is available online as Supplementary Material). Briefly, heparinized harvested bone marrow was filtered through a 170 – 260 micron blood filter (Baxter, Deerfield, IL, USA) into a 600-mL transfer pack. A volume of Hetastarch (6%; Abbott, Abbott Park, IL, USA) equal to 20% of the bone marrow volume was added and centrifuged for 7 min at $50 \times g$. The white cell-rich plasma was removed using a plasma extractor (Fenwal Blood Technologies, Lake Zurich, IL, USA) and counted using an automated hematology analyzer (AcT Diff 2; Beckman Coulter, Fullerton, CA, USA). There were two conditions in which a second Hetastarch separation was performed. (a) If the White Blood Cell (WBC) recovery was $< 80\%$, the pelleted red cells were resuspended in saline – 0.5% human serum albumin (HSA) to the original volume and admixed with additional Hetastarch (20% v/v). The process was repeated and the white cell-rich fractions pooled. (b) If the red cell mass of the white cell-rich fraction exceeded 25 mL, Hetastarch (20% v/v) was added to the white cell-rich fraction and the process was repeated.

Immunomagnetic separation of CD34⁺ cells from red cell-depleted bone marrow

The white cell-rich fraction was washed once with saline – 0.5% HSA, resuspended in 60 mL 0.5% HSA plus 10 mL intravenous immune globulin (human, 5% stock) and incubated at room temperature for 10 min. The product volume was then brought to 800 mL with Isolex buffer (phosphate-buffered saline, 0.5% HSA) and processed on an Isolex 300i (software revision 2.5) for CD34 enrichment according to the manufacturer's instructions. Release testing (Gram stain, endotoxin, CD34 number, purity and viability) was performed and reported prior to product administration. Release criteria were: Gram stain negative, endotoxin < 5.0 EU/kg patient body weight (Endosafe PTS; Charles River, Charleston, SC, USA), CD34 purity $> 56\%$, CD34 viability $> 98\%$ (7AAD exclusion), total CD34 $> 1 \times 10^6$. Table I gives the elapsed times required to perform the entire procedure for a separation in which no unexpected difficulties were encountered.

Bone marrow aspirates for characterization of CD34⁺ cells

Bone marrow aspirates were harvested from the humerus of orthopedic patients ($n = 5$) mainly with shoulder instability. Subject ages ranged from 22 to 90 years of age (mean 53.4). Samples were delivered to the laboratory without patient identifiers. Bone marrow mononuclear cells were isolated by density-gradient centrifugation using Ficoll/Hypaque (1.077 g/mL; Sigma Diagnostics, St. Louis, MO, USA) at $400 \times g$ for 30 min at room temperature. After centrifugation the buffy coat was collected and washed three times with phosphate-buffered saline (PBS-A) at $700 \times g$ for 7 min. WBC counts were performed before and after processing using an AcT10 hematology analyzer (Beckman Coulter).

Flow cytometry for clinical CD34 enumeration

Flow cytometry was performed using a commercially available single platform lyse/no-wash assay (Stem-Kit, Beckman Coulter) according the manufacturer's instructions. Data were

acquired on a 4-color Beckman Coulter Epics XL cytometer, which was calibrated daily with Flow-Check and Flow-Set beads (Beckman Coulter). CD34 reference cells (low, normal and high controls) (Streck Laboratories, Omaha, NE, USA) were run with each sample as a positive control of known CD34 content. Data were acquired immediately and analyzed in real-time using System II software (Beckman Coulter) and a modification of the Sutherland ISHAGE method (1,2). A representative sample was re-analyzed (Figure 1) using VenturiOne software (Applied Cytometry Systems, Sheffield, UK).

Flow cytometry for characterization of heterogeneity of CD34⁺bone marrow cells

The mononuclear cell pellet from Ficoll/Hypaque-separated bone marrow was incubated with decompartmented mouse serum (2 μ L) for 5 min at room temperature to block non-specific antibody binding. Cells were then stained with monoclonal antibodies against CD105 – fluorescein isothiocyanate (FITC) (Fitzgerald Industries Int., Concorde, MA, USA), CD73 – phycoerythrin (PE), CD45 – Allophycocyanin (APC)-Cyanine(Cy7) (BD Biosciences, San Jose, CA, USA), CD34 – Energy Coupled Dye (ECD), CD90 – Texas Red(PE)-Cy5, CD117 – PE-Cy7 (Beckman Coulter) and CD133 APC (Miltenyi Biotec Inc., Auburn, CA, USA) for 20 min on ice. Following staining, the cells were washed and fixed for 20 min with 2% methanol-free formaldehyde and permeabilized with saponin (PBS with 0.5% Bovine Serum Albumin (BSA), 0.1% saponin) for 10 min at room temperature. Prior to acquisition, 4 μ L 4',6-diamidino-2-phenylindole (DAPI) were added per 10⁶ cells, as a marker of nucleated cells. Cells with less than diploid DNA content were excluded from the analysis. Five to 10 million cells per sample were acquired on a Beckman Coulter CyAn ADP cytometer and the data were spectrally compensated and analyzed offline using VenturiOne rare-event analytical software.

Results

Erythrocyte depletion by Hetastarch sedimentation

Prior to immunomagnetic separation, harvested bone marrow ($n = 10$) was depleted of erythrocytes using Hetastarch sedimentation (Table II). The hematocrit of harvested bone marrow averaged $29.3 \pm 4.3\%$ (mean $\pm$ SD). Hetastarch sedimentation was effective in depletion of erythrocytes (mean depletion 91.2%) but not without some loss of CD34⁺ cells (mean recovery 68.3%). Two cell-processing runs (samples 2 and 4; Table II) were aborted following Hetastarch sedimentation because of insufficient total cell count.

Isolex selection of erythrocyte-depleted bone marrow

Erythrocyte-depleted bone marrow cells ($n = 8$) were washed once in PBS with 0.5% HSA, resuspended in 800 mL PBS HSA and processed on the Isolex according to the manufacturer's instructions for leukapheresis products. The instrument tolerated the erythrocyte burden well (maximum of 2×10^{11} Red Blood Cell (RBC), sample 6). CD34 purity after CD34 selection averaged $64.1 \pm 17.2\%$ and CD34 recovery was $44.3 \pm 26.1\%$ (Table III). CD34 enrichment averaged 121-fold $\pm$ 44, compared with the erythrocyte-depleted product with an average yield of $5.0 \pm 2.7 \times 10^6$ CD34⁺ cells (mean $\pm$ SD). Both the total yield ($r^2 = 0.74$, $P = 0.006$) and the purity ($r^2 = 0.81$, $P = 0.005$) of the final product were closely correlated by linear regression with the number of CD34⁺ cells in the

harvested bone marrow, indicating the importance of the quality of the original starting material. Neither patient age nor RBC contamination of the harvested bone marrow (a surrogate for hemodilution, as peripheral blood aspiration increases the hematocrit) correlated with CD34 yield in the final product.

Heterogeneity of bone marrow CD34⁺ cells

In addition to hematopoietic progenitor cells, bone marrow contains rare populations of primitive and/ or non-hematopoietic (CD45⁻) stem cells, including endothelial stem cells. Bone marrow cells selected on the Isolex would also be expected to contain these populations, providing that they were CD34⁺. As it was not possible to investigate this heterogeneity directly on clinical products, we examined freshly isolated bone marrow aspirate mononuclear cells from orthopedic patients by multiparameter flow cytometry, first gating on total CD34 cells and then probing for the expression of stem/progenitor markers CD90, CD117 and CD133 and mesenchymal stromal cell markers CD73 and CD105. Figure 2 shows the gating strategy used for this analysis. Population means and standard deviations are shown on the figure and results are detailed in the figure legend. The results indicated that the CD34⁺ fraction of bone marrow contained non-hematopoietic stem cells that may include mesenchymal and endothelial progenitor populations.

Discussion

Isolation of CD34⁺ cells from bone marrow for clinical use dates to the early 1990s (3). The commercial CellPro CEPRATE immunomagnetic device was introduced a few years later (4,5), when bone marrow was still the dominant source of hematopoietic progenitor cells. As aspirated bone marrow has a significant hematocrit, erythrocyte depletion was required prior to CD34 enrichment. This was usually accomplished by preparation of a bone marrow buffy coat using a leukapheresis machine. In contrast to these early studies, the Baxter Isolex 300i, a closed-system immunomagnetic separation device, was developed for CD34 selection from mobilized leukapheresis products, which have minimal erythrocyte content. The Isolex was first commercially released in 1999 with FDA licensing for tumor purging in the context of autologous peripheral stem cell transplantation. As such, it was designed to enrich CD34⁺ cells from cytokine or cytokine/chemotherapy-mobilized leukapheresis products.

The field of regenerative medicine has spurred renewed interest in the use of bone marrow as a source of multipotent stem cells. In the case of small volume separations, whole bone marrow can be separated directly on the Isolex without significant interference of the far more numerous erythrocytes (6). Larger scale applications, such as the one detailed here, require red cell depletion. Requirements for optimal erythrocyte depletion include: (a) a closed system compatible with current good manufacturing practices; (b) sufficient depletion of erythrocytes; (c) acceptable white blood cell recovery; and (d) minimal processing time. The buffy coating procedure used for CD34 selection during the early 1990s fulfilled points (a) and (b). Although the efficiency of red blood cell depletion and leukocyte recovery in the buffy coat was not usually reported, our own studies in the same era using a bone marrow buffy coat prior to centrifugal elutriation reported excellent

erythrocyte depletion and a WBC yield of about 44% (7,8). In the present study, we opted for Hetastarch sedimentation, which has been used widely for erythrocyte depletion of bone marrow products in the context of ABO-mismatched allogeneic transplantation and has been reviewed by Spitzer (9). Leukocyte recoveries as high as 87% have been reported when sedimentation is performed at unit gravity (10), but this typically requires a sedimentation time of 45 min. The advantages of Hetastarch sedimentation include simplicity, closed system processing and infusion compatibility.

Our study had the unique requirement for intra-operative product preparation during which time the patient is held under general anesthesia. In an effort to minimize the processing time required for erythrocyte depletion, we substituted low speed centrifugation ($50 \times g$) for gravity sedimentation, reducing sedimentation time from 45 to 7 min. The sole disadvantage appeared to be a variable loss of CD34⁺ cells during this procedure, which ranged from 0 to 68% (mean 31.7%; Table II) in our series. Based on an estimate of the maximal cell load that could be sustained by the Isolex, we specified that the residual erythrocyte packed cell volume after Hetastarch must not exceed 25 mL. This necessitated a second round of sedimentation in five of 10 cases, increasing the processing time not resulting in a lower CD34 recovery.

Following erythrocyte depletion, products were processed on the Isolex according to the method recommended for leukapheresis products. In this series, both the white blood cell count ($9.8 \pm 2.9 \times 10^3$ cells/ μ L) and the CD34 content ($0.5 \pm 0.2\%$) of harvested bone marrow was lower than expected from prior experience with allogeneic bone marrow donors (17.7×10^3 and 0.71%, respectively) (11). This discrepancy may reflect both the older age and general health of the patients recruited for this study. CD34 recovery ($44 \pm 26\%$) was consistent with our experience with leukapheresis products, but the average CD34 purity ($64.1 \pm 17.2\%$) was lower than obtained with peripheral blood products. The final CD34 recovery and purity were highly dependent on the CD34 content of the harvested bone marrow, emphasizing that successful immunomagnetic cell separation requires high-quality input material.

Detailed flow cytometric analysis of CD34⁺ bone marrow cells revealed the heterogeneity of this compartment, including the presence of a sizable fraction of CD45⁻ cells ($17.0 \pm 4.7\%$) (Figure 2, H). Such cells would be difficult to detect with the ISHAGE protocol, which uses CD45 gating chiefly to distinguish between cells and debris. In the ISHAGE assay CD45⁻ cells are intermixed with debris and difficult to quantify. In the present analysis, we used DNA staining to resolve nucleated cells from CD45⁻ debris, allowing the accurate enumeration of CD45⁻ cells within the CD34 compartment. By acquiring 5 – 10 million events, taking care to remove doublets and rare autofluorescent cells (0.4%), both of which would interfere with the detection of very rare events, we were able to detect CD34 subsets that would be contained within a CD34-selected bone marrow product. These subsets included CD34⁺ cells bearing the mesenchymal stromal cell-associated markers CD73 and CD105, on which CD45 expression ranged from bright to negative. Although CD34 is not a classic mesenchymal stromal cell marker, CD73 and CD105⁺ bone marrow cells may be analogous to the rare subset of adipose stem cells that are transitional between CD34⁻ pericytes and CD34⁺ supra-adventitial adipose stromal cells (12). CD105, a component of

the transforming growth factor receptor complex, has also been reported on primitive CD34⁺ hematopoietic cells (13). Further, significant proportions of CD34⁺ CD90⁺ (22.4 ± 9.5%) and CD34⁺ CD117⁺ (15.9 ± 5.7%) bone marrow cells were CD45⁻, suggesting non-hematopoietic or very early hematopoietic stem cells. These rare subpopulations have not been well characterized but are likely to include multipotent non-hematopoietic stem/progenitor cells, which are of interest for both regenerative and anti-inflammatory therapy.

In the context of cardiac therapy, as well as other regenerative applications, the dose and purity of CD34⁺ cells required for therapeutic effect is not known. Therefore, product suitability must be determined empirically in each therapeutic context by dose escalation. Although the results available to date indicate that administration of purified autologous CD34⁺ cells is well tolerated (14,15), efficacy remains to be demonstrated. The results presented here provide a reproducible methodology applicable to intra-operative cell processing that will be of value to investigators continuing to explore the therapeutic applications of bone marrow-derived CD34⁺ cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

CD34 content of bone marrow at harvest, after erythrocyte depletion and after CD34 enrichment on the Isolex. Data from a representative subject were analyzed according to the ISHAGE protocol. Two-parameter histogram columns (left to right) show detection of stem count beads, detection of CD45⁺ white blood cells, detection of CD34⁺ cells among white blood cells, verification of homogeneity of CD45 expression on CD34⁺ cells, verification of light scatter properties of CD34⁺ cells, and verification of viability (7AAD exclusion) of CD34⁺ cells. In this sample, 0.86% of bone marrow cells were CD34⁺ (98.6% viability at time of assay). Erythrocyte depletion resulted in a modest increase in the proportion of CD34⁺ cells. CD34⁺ cells of 99.9% viability constituted 89.1% of white blood cells in the immunomagnetically separated product, representing a greater than 100-fold enrichment. CD34 recovery was 32.5% of harvested bone marrow and 45.9% of erythrocyte-depleted bone marrow.

Figure 2.

Characterization of the CD34 content of aspirated bone marrow. Bone marrow aspirates, obtained from five subjects undergoing orthopedic surgery, were separated on a Ficoll/Hypaque gradient prior to staining. Five to 10 million events were acquired. A typical analysis is shown, with mean $\pm$ SD for all five subjects indicated in the histograms. The top two rows of histograms show the gating strategy used to eliminate multiple sources of artifact encountered in rare event problems. From left to right: forward scatter pulse analysis (A) removes cell clusters; the DNA stain DAPI (B) removes erythrocytes, apoptotic cells

and subcellular debris; low light scatter events are removed (C); a compound gate (D, E and F) removes autofluorescent events, which account for 0.4% of cells with $\geq 2N$ DNA. Third row (G, H): CD34⁺ cells are detected among non-autofluorescent singlet events with DNA content $\geq 2N$, 17% of which are CD45⁻ compared with an isotype control (data not shown). Fourth row (I–M): mesenchymal (CD105, CD73) and adult stem/progenitor cell (CD90, CD117, CD133) marker expression on CD34⁺ bone marrow cells. Fifth row: proportion of CD45⁻ cells among each CD34⁺ subset defined in row 4.

Table I Elapsed time for erythrocyte depletion, CD34 selection and release testing.

Procedure	Elapsed time
Product transport from operating room to laboratory	20 min
Product accession	18 min
Erythrocyte depletion and wash (two rounds of Hetastarch)	55 min
Isolex separation	3 h 2 min
Release testing and product transport from laboratory to operating room	1 h 50 min
Total elapsed time	6 h 5 min

Table II

Erythrocyte depletion of harvested bone marrow. The products are ordered according to CD34 content of the harvested bone marrow.

Sample ID	Harvest			Hetastarch			
	Volume (mL)	TRBC	TCD34	TRBC	TCD34	% RBC Dep	% CD34 Rec
1	345	1.2E+12	8.3E+06	1.4E+11	8.4E+06	88.0	101.2
2	370	1.2E+12	8.9E+06	1.3E+11	7.4E+06	89.3	82.9
3	265	7.0E+11	9.5E+06	4.6E+10	7.0E+06	93.4	73.1
4	319	9.9E+11	1.1E+07	4.1E+10	4.9E+06	95.9	42.8
5	192	5.9E+11	1.3E+07	7.1E+10	7.4E+06	88.1	55.4
6	501	2.1E+12	1.7E+07	2.0E+11	1.4E+07	90.4	83.6
7	444	1.6E+12	1.8E+07	1.2E+11	5.7E+06	92.3	32.1
8	479	1.2E+12	1.9E+07	1.4E+11	1.4E+07	88.4	72.4
9	348	1.4E+12	3.0E+07	1.1E+11	2.1E+07	91.9	70.9
10	330	1.3E+12	3.5E+07	7.2E+10	2.4E+07	94.4	68.6
Mean	359	1.2E+12	1.7E+07	1.1E+11	1.1E+07	91.2	68.3
Min	192	5.9E+11	8.3E+06	4.1E+10	4.9E+06	88.0	32.1
Max	501	2.1E+12	3.5E+07	2.0E+11	2.4E+07	95.9	101.2
SD	95	4.3E+11	9.0E+06	4.9E+10	6.7E+06	2.8	20.3
CV(%)	26.5	35.0	53.2	46.2	58.8	3.1	29.7

TRBC, total red blood cells; TCD34, total CD34⁺ cells; RBC Dep (depletion), percentage of original RBC content remaining after Hetastarch sedimentation; CD34 Rec, CD34 recovery after Hetastarch sedimentation; SD, standard deviation; CV, coefficient of variation.

Table III

Isolex selection of erythrocyte-depleted bone marrow. The product was then selected on the Baxter Isolex according to the manufacturer's instructions for leukapheresis products. In two cases the selection was not performed because of low CD34 recovery in the harvested bone marrow. The products are ordered according to CD34 content of the harvested bone marrow.

Sample ID	Hetastarch			Product			
	TNUC	TCD34	% CD34	TNUC	TCD34	% CD34	CD34 Rec
1	3.2E+09	8.4E+06	0.26	5.2E+06	2.0E+06	38.9	24.3
2	1.4E+09	7.4E+06	0.54	Selection aborted			
3	2.1E+09	7.0E+06	0.34	5.9E+06	3.0E+06	50.9	42.8
4	1.0E+09	4.9E+06	0.48	Selection aborted			
5	1.1E+09	7.4E+06	0.67	5.8E+06	3.0E+06	52.8	40.9
6	2.6E+09	1.4E+07	0.54	4.7E+06	2.9E+06	60.9	20.6
7	1.5E+09	5.7E+06	0.38	7.6E+06	5.9E+06	78.6	104.2
8	1.7E+09	1.4E+07	0.78	7.7E+06	6.2E+06	80.5	45.7
9	1.8E+09	2.1E+07	1.21	1.1E+07	9.8E+06	89.1	45.9
10	3.8E+09	2.4E+07	0.63	1.2E+07	7.1E+06	61.2	29.9
Mean	2.0E+09	1.1E+07	0.58	7.4E+06	5.0E+06	64.1	44.3
Min	1.0E+09	4.9E+06	0.26	4.7E+06	2.0E+06	38.9	20.6
Max	3.8E+09	2.4E+07	1.21	1.2E+07	9.8E+06	89.1	104.2
SD	9.0E+08	6.7E+06	0.27	2.6E+06	2.7E+06	17.2	26.1
CV (%)	45.0	58.8	46.5	35.3%	54.2%	26.8	59.0

TNUC, total nucleated cells; CD34 Rec, CD34 recovery as a percentage of CD34⁺ cells present in the erythrocyte-depleted product, E+: Base 10 exponent.