RESEARCH ARTICLE

Neuraminidase enhances *in vitro* expansion of human erythroid progenitors

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ABSTRACT. ***Background:*** **In spite of recent key improvements, *in vitro* mass production of erythrocytes from human stem cells is still limited by difficulties in obtaining sufficient numbers of erythroid progenitors. In fact, such progenitors are as scarce in the bone marrow as in peripheral blood.** ***Study design and Methods:*** **We used a two-step culture model of human cord blood-derived erythroid progenitors in the presence or absence of high-purity neuraminidase, in a serum-free, defined culture medium. Granulocytic and megakaryocytic progenitor cell expansions were also studied.** ***Results:*** **We show that significant enhancement of erythroid cell generation is obtained when CD34$^+$ human hematopoietic progenitors are cultured in the presence of neuraminidase. Interestingly, in so doing, expanded red cell progenitors remained erythropoietin-dependent for further expansion and survival, and cells thus generated displayed a normal phenotype. Moreover, the activity of neuraminidase on these cells can be reversed by simple cell washing. Finally, growth of cells of the other myeloid lineages (granulocytes and megakaryocytes) is either decreased or unchanged in the presence of neuraminidase.** ***Conclusion:*** **This specific feature of neuraminidase, that of stimulation of human red cell progenitor proliferation, provides a safe technique for producing greater numbers of *in vitro*-generated red blood cells for both basic research and transfusion use.**

Key words: neuraminidase, cell expansion, erythroid progenitors, human primary cells, *in vitro* cultures

Sialic acids (SIAS) are sugars expressed at the surface of all cell types. Abnormalities of SIAS presence and distribution on cell membranes are observed in various pathological conditions including infectious diseases, severe inflammatory conditions, malignancies and genetic disorders [1, 2]. Extracellular neuraminidases (sialidases) are important virulence factors for many bacterial pathogens such as *Clostridium perfringens* and *Pneumococcus*, or for viruses such as *Influenza* viruses. Neuraminidases can cleave terminal SIAS residues in O-linked and N-linked glycans of cell membrane proteins [3]. Notably, the red blood cell (RBC) surface contains a cryptic carbohydrate antigen covered by SIAS residues, called *Thomsen-Friedenreich antigen* (T-antigen), which can be unmasked through the action of neuraminidases [4, 5].

The effects of bacterial neuraminidases on hematopoiesis remain mostly unexplored, in spite of the fact that some previous reports have described a role for sialylation on granulocyte and platelet generation. SIAS are present on various glycan types in α2-3, -6 or -8 linkages, which are generated by a family of 18 sialyltransferase genes. Interestingly, the sialyltransferase ST6Gal-1, an enzyme up-regulated in pathological conditions such as cancer and acute systemic inflammatory responses [6, 7] has been shown to play a role in granulocyte homeostasis and myeloid cell differentiation. Actually, ST6Gal-1-deficient mice display increased granulopoietic capacity [8], and soluble ST6Gal-1 may serve to modulate myelopoiesis [9]. Furthermore, it has been reported that sialylation also influences platelet generation since a defect in α2-3 sialylation is associated with defective megakaryopoiesis [9]. Finally, desialylation of membrane proteins by neuraminidase, unmasking the T-antigen on RBCs and platelets [4, 10], contributes to hemolysis and thrombocytopenia [5, 11, 12].

We wondered whether neuraminidase affected the expansion of erythroid progenitors (EPs) from purified human

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primary progenitor cells present in cord blood, and if it was involved in the mechanism of anemia observed in inflammatory conditions linked to pathogens producing neuraminidase. With that aim, we studied *in vitro* the effects of neuraminidase in a two-step culture model of human erythroid cell expansion. We observed significant alterations in *in vitro* cell generation when hematopoietic progenitors were exposed to neuraminidase. Expansion of red cell progenitors was enhanced and this remained EPO-dependent; cells thus generated displayed a normal phenotype. Moreover, cellular growth of the other myeloid lineages was not improved. This clear-cut feature of neuraminidase activity on EPs provides a base from which to boost *in vitro* production of human RBCs.

METHODS

Isolation and immuno-labelling of CD34⁺ cells

Normal cord blood was collected according to institutional guidelines and after informed consent of the mothers. $CD34^+$ cells were then purified as previously described [13] and their percentage was determined using allophycocyanin (APC)-conjugated antibody (Beckman Coulter Inc., Villepinte, France) after analysis with a FACSCalibur flow cytometer (Becton Dickinson, Le-Pont-de-Claix, France). For all cultures, the percentage of $CD34^+$ cells in the initial inputs was always $>90\%$.

Cell cultures

For the red cell lineage cultures, $CD34^+$ cells were plated in serum-free IMDM (Invitrogen Gibco) with 15% BIT 9500 (StemCell Technologies, Vancouver, Canada). A cytokine mixture containing recombinant human interleukin-3 (rhIL-3, 10 ng/mL) + rhIL-6 (10 ng/mL) + rhStem Cell Factor (rhSCF, 25 ng/mL) (Amgen, Thousand Oaks, USA) was added. Cells were initially grown for seven days, then cultured again for one to eight days in IMDM containing 15% BIT 9500 + rhIL-3 + rhIL-6 + rhSCF + rhErythropoietin (rhEPO, 2 U/mL). When indicated in the text and figures, rhEPO addition to the cultures was omitted at day 0 to test the EPO-autonomy hypothesis of red progenitor cell expansion in presence of neuraminidase.

For granulocytes and megakaryocytes (MKs), the same basic medium was used, and complemented with either rhIL-3 (10 ng/mL) + rhSCF (25 ng/mL) + rhFlt3-ligand (10 ng/mL) + rhGranulocyte-colony stimulating factor (rhG-CSF, 20 ng/mL), or thrombopoietin (TPO) agonist AF13948 (Sigma-Aldrich) (25 nM) + rhSCF (10 ng/mL once at cell input), respectively. Granulocytes were then cultured for 13-15 days, with the whole cytokine medium being renewed every three to four days, whereas MKs were cultured for 13 days in the presence of the TPO peptide only.

When required, neuraminidase from *Vibrio cholerae* type II (Sigma-Aldrich, Lyon, France) was added to the cultures at a final concentration of 1 or 20 mU/mL. Of the neuraminidases, that from *Vibrio cholerae* was chosen since its optimal pH is compatible with cell culture media and because it cleaves terminal SIAS with a large specificity spectrum of action (α2-3, -6 or -8 linkages) [5, 12]. The neuraminidase used was a highly purified, preservative-free product (purity $>98\%$ according to the manufacturer's indications), with a specific activity of 8-24 units/mg protein. All cell counts were performed on Malassez slides using trypan blue exclusion. Overall cell culture conditions are schematized in *figure 1* .

Colony-forming cell potential

The colony-forming cell (CFC) potential was evaluated by plating 400 cells per mL in methylcellulose medium (MethoCult H4230, StemCell Technologies), supplemented with cytokines: rhIL-3 (10 ng/mL) + rhSCF (25 ng/mL) + rhG-CSF (20 ng/mL) + rhGranulocyte-macrophage (GM)-CSF (5 ng/mL) + rhEPO (2 U/mL). Colonies were scored after a 15-day growth at 37°C, in a fully humidified atmosphere containing 5% CO_2.

Cell phenotype analysis and evaluation of apoptosis

The antibodies used for the phenotypic analysis of the cells were phycoerythrin (PE)-conjugated mouse monoclonal antibodies (mAB) anti-CD235a for erythroid cells, PE-conjugated mAB against CD14 and CD15 for myelomonocytic cells, and FITC-conjugated mAB against CD41a and CD42b for MKs. Isotype-matched antibodies were used as controls. All antibodies were from Beckman Coulter. The detection of T-antigen on the cell surface was determined by flow cytometry using fluorescein-isothiocyanate (FITC)-conjugated *Arachis hypogaea* lectin (Sigma-Aldrich). Briefly, cells were washed in PBS (Invitrogen Gibco) and stained with the lectin (diluted 1/10,000 in PBS) for 30 min. at 4°C. Samples were then suspended in 500 µL PBS and analyzed on a FACSCalibur flow cytometer. AnnexinV-FITC-positive events, which confirms phosphatidylserine-exposure on cultured progenitors, was tested using the Apoptest-FITC kit (DAKO, Trappes, France).

Statistical analyses

Quantitative data are expressed as arithmetic mean ± standard error (SEM), and categorical data as numbers with percentages. Statistical analyses were performed with the GraphPad Prism software v5.03 (La Jolla, CA, USA). Values of $p<0.05$ were considered statistically significant.

RESULTS

Neuraminidase enhances in vitro expansion of erythroid progenitors in a dose-dependent manner

We first assessed the effect of neuraminidase on erythroid cell expansion. With that aim, two-step cultures were performed using cord blood-derived $CD34^+$ cells as previously described [13] in the presence of either 1 or 20 mU/mL of neuraminidase, or in absence of neuraminidase as a control. Under our conditions, rhEPO was added at day 7 to allow differentiation. The first three experiments conducted allowed us to determine whole cell production at day 13 of the cultures, corresponding to the moment of final cell differentiation of most erythroid progenitors. Intriguingly, when neuraminidase was added at day 1, massive amplification of red cell progenitors was observed. Whereas untreated cells were 457 ± 61-fold

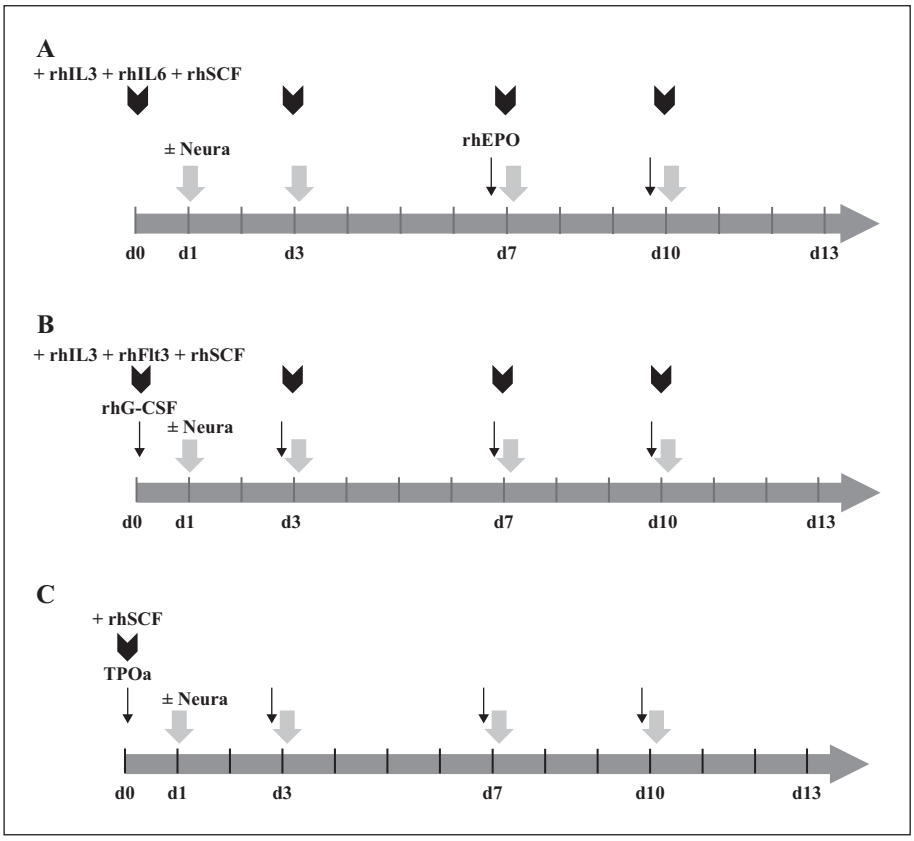

Figure 1

Procedures of *in vitro* culture expansion from progenitors of the various myeloid cell lineages. (A) Erythroid cell expansion. A two-time method was used, as described [27]: cells were initially cultured in the presence of three cytokines (rhIL-3 + rhIL-6 + rh SCF, added at days 0, 3, 7, 10 as indicated by thick dark-grey arrowheads). rhEPO was added twice, at days-7 and -10 (thin black arrows). **(B)** Granulocytic cell expansion. Cells were cultured in the presence of rhIL-3 + rhIL-6 + rhSCF (thick dark-grey arrowheads) and G-CSF (thin black arrows), added at days 0, 3, 7, 10. **(C)** Megakaryocytic cell expansion. Cells were cultured in the presence of rhSCF (one addition at day 0, thick dark-grey arrowheads), and TPO receptor agonist added at days 0, 3, 7 and 10 (thin black arrows). In all cases, when added, neuraminidase was added to the cultures at days 1, 3, 7, and 10 (as indicated by thick light-grey arrows). Cultures were stopped at day 13.

expanded when compared to the number of cells seeded at day 0, and progenitor cells cultured in the presence of 1 or 20 mU/mL of neuraminidase were 1651 ± 296-fold or 3067 ± 494-fold expanded, respectively (*figure 2A*). Such enhancement of cell expansion was significant for both neuraminidase dose treatments ($p = 0.0168$ for 1 mU/mL neuraminidase compared to untreated cells; $p = 0.0063$ for 20 mU/mL neuraminidase compared to untreated cells). Other representations of raw data are in *figures 3A, B*. Thus, neuraminidase-treated/untreated expansion ratios of red cell progenitors at day 13 were 3.66 ± 0.92 for 1 mU/mL neuraminidase treatment (Neura 1) and 7.03 ± 1.83-times enhanced for 20 mU/mL neuraminidase treatment (Neura 20), respectively, when compared to the untreated cells (*figure 3C*).

Since the addition of 20 mU/mL neuraminidase always resulted in the best expansion of EPs, further culture studies were performed using that concentration alone, compared to non-treated controls. Five additional experiments were then performed to explore further the expansion of hematopoietic progenitors in the presence or absence of 20 mU/mL of neuraminidase from day 1 (*figure 1A*). Expansion rates were determined four times during the cultures and were: 1.16 ± 0.2 at day 3, 2.62 ± 1.2 at day 7, 6.09 ± 4.5 at day 10 and 5.21 ± 2.5 at day 13 (*figure 2B*). This demonstrates that the red cell progenitor expansion increase observed with neuraminidase, under our conditions, takes place early during the culture, before the additionof rhEPO. When the results are reported as cell number raw data (line graphs, *figures 2C, D*), the number of EPs obtained at day 7 was $1.16 \pm 0.63 \times 10^6$ with non-treated cells, whereas it reached $2.67 \pm 1.40 \times 10^6$ with neuraminidase-treated cells (*figure 2C*; values for day 7). This phenomenon continued during the second EPO-dependent phase of erythropoiesis (*figure 2D*): the cell number obtained at day 13 was $6.73 \pm 2.9 \times 10^6$ for untreated and $13.7 \pm 6 \times 10^6$ for neuraminidase-treated cells. On the whole, in that series of experiments, the pre-EPO initial phase expansion of cells was 2.3-times enhanced in presence of neuraminidase when compared to untreated cells, and then a further twice enhanced during the second phase of the culture with rhEPO. It must be emphasized that the addition of EPO at day 0 of culture resulted in a remarkable reduction in neuraminidase-induced erythroid progenitor expansion ratios throughout the culture (two independent representative experiments are shown in *figure 4A*).

We also examined whether differences in growth observed between treated and untreated cells could be due to variations in the evolution of the $CD34^+$ cell population during culture. As shown in *figure 2E*, the evolution of the percentage of $CD34^+$ cells between day 1 and day 15 of culture was similar with neuraminidase-treated and untreated cells (1-way ANOVA; $p = 0.9646$).

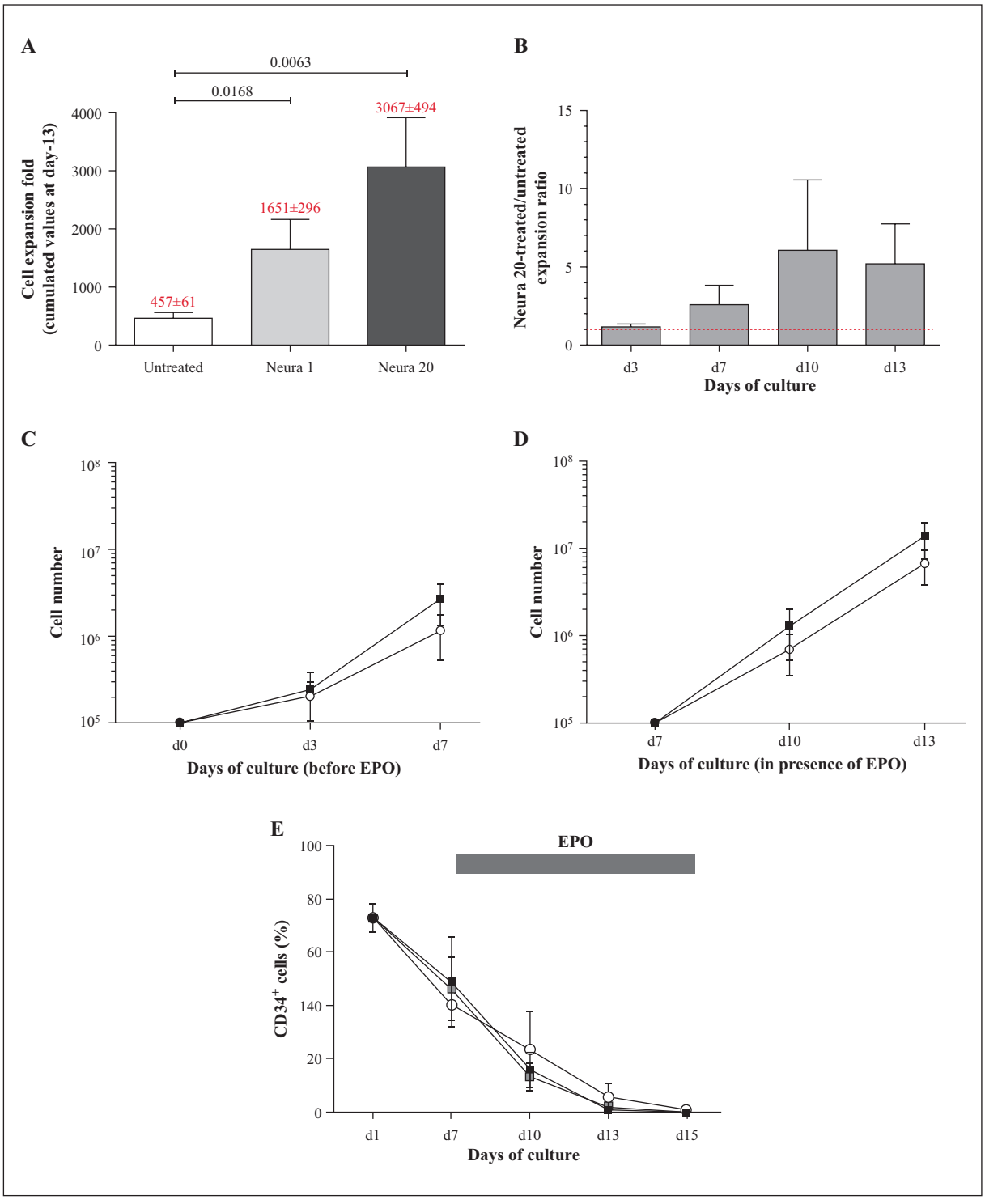

Figure 2

Expansion of red cell progenitors cultured with or without neuraminidase. (A) Expansion folds: red cell progenitors cultured without (white bar) or with neuraminidase 1 mU/mL (Neura 1; light-grey bar) or 20 mU/mL (Neura 20; dark-grey bar) added at day 1. At day 13, cells were counted and cell expansion fold determined. Statistical analysis was done using Student's *t* test. **(B)** Expansion ratios calculated from erythroid progenitors cultured in the presence of neuraminidase 20 mU/mL (Neura 20-treated) or untreated. Cells were counted at day 3, day 7, day 10 and day 13 during the culture and then Neura20-treated/untreated expansion ratios were calculated. Normal expansion of red cell progenitors, without neuraminidase (baseline corresponding to a value of 1 is indicated as the red dashed line. **(C)** Cumulated numbers of cells obtained during the culture of non-treated (white circles) or 20 mU/mL neuraminidase-treated (black squares) erythroid progenitors between day 0 and day 7 (first phase of the culture; before addition of EPO), if 100,000 CD34+ cells were seeded at day 0. **(D)** Cumulated numbers of cells obtained during the culture of non-treated (white circles) or 20 mU/mL neuraminidase-treated (black squares) erythroid progenitors between day 7 and day 13 (second phase of the culture, i.e. after addition of EPO) if 100,000 CD34+ cells were seeded at day-7. **(E)** Determination of the % of $CD34^+$ cells during the day 0 to day 15 culture period. RCPs were cultured without neuraminidase (white circles) or with neuraminidase 1 mU/mL (grey squares) or 20 mU/mL (black squares). At day 7, EPO was added (represented by the horizontal dark-grey bar). All the data (A-E) represented are the means ± SEM of 3-5 independent determinations.

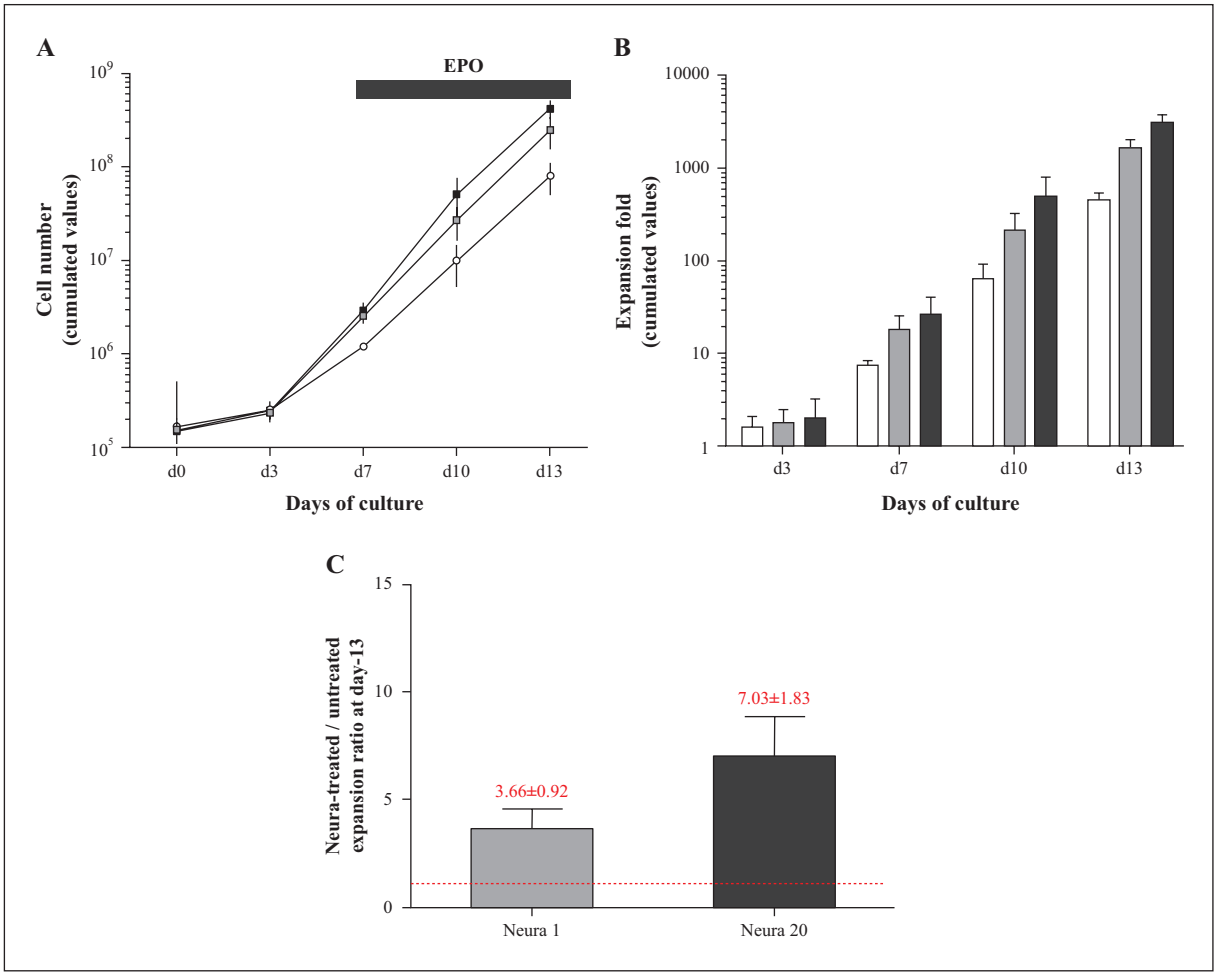

Figure 3

Expansion of RCPs cultured with or without neuraminidase. Red cell progenitors cultured at day 0 in complete liquid medium were untreated or treated with neuraminidase 1 mU/mL or 20 mU/mL at day 1; at day 13, cells were counted. Data represented are means ± SEM of the values obtained from three independent experiments. Cumulated values are represented. **(A)** Representation of the cell number obtained as line graphs, with cells untreated (white square), cells treated with neuraminidase 1 mU/mL (light-grey square) or 20 mU/mL (dark-grey square). At day 7, EPO was added (represented by the horizontal dark-grey bar). **(B)** Representation of expansion fold results as histograms: cells were untreated (white bar) or treated with neuraminidase 1 mU/mL (light-grey bar) or 20 mU/mL (dark-grey bar). To simplify the figure, addition of rhEPO represented by the horizontal dark-grey bar, is omitted on this graph. **(C)** Expansion ratios calculated at day 13, after culture of red cell progenitors with neuraminidase 1 mU/mL (light-grey bar) or 20 mU/mL (dark-grey-bar). Data represented are means ± SEM of the values obtained (n = 3). The baseline, indicated as a red dashed line, corresponds to the value of 1 for non-treated progenitors.

Neuraminidase-mediated expression of the Thomsen-Friedenreich antigen (T-antigen) on erythroid cells in culture is reversible.

We first verified that the addition of neuraminidase was in fact able to unmask the T-antigen at the surface of all treated erythroid cells in culture. As soon as neuraminidase was added (from day 1 to day 13; data for T-antigen detection are indicated from day 4 to day 13), we observed expression of the T-antigen on 100% of treated cells. We then addressed whether removing neuraminidase from the cultures could suppress the expression of cell surface T-antigen, through the sialyltransferase activity of hematopoietic cells, which is capable of resialylating major surface glycoproteins [14]. When cells cultured in the presence of neuraminidase were washed twice, we observed that the surface T-antigen quickly became undetectable if these cells were cultured again in the absence of neuraminidase (*figure 5*). Untreated cells always remained negative for the presence of T-antigen, throughout the culture (data not shown). This important observation provides evidence of the full reversibility of this effect of neuraminidase on *in vitro* expanded erythroid cells.

Red progenitor cell expansion in the presence of neuraminidase is not EPO-autonomous

We wondered whether RBC expansion, during the second phase of culture, could be related to neuraminidase-mediated desialylation of the EPO/EPO receptor coupling molecules, given that it has been shown that the glycosylation level can affect the binding of EPO to its receptor [15]. In this case, autonomy of the cells as regards EPO might be observed. As shown in *figure 6*, no change in untreated *versus* neuraminidase-treated cell expansion from day 7 culture cells was detected, indicating that growth and differentiation of cells after the first culture step remained EPO-dependent, even in the presence of neuraminidase.

In order to determine if neuraminidase-dependent cell growth was limited to the red cell lineage or concerned

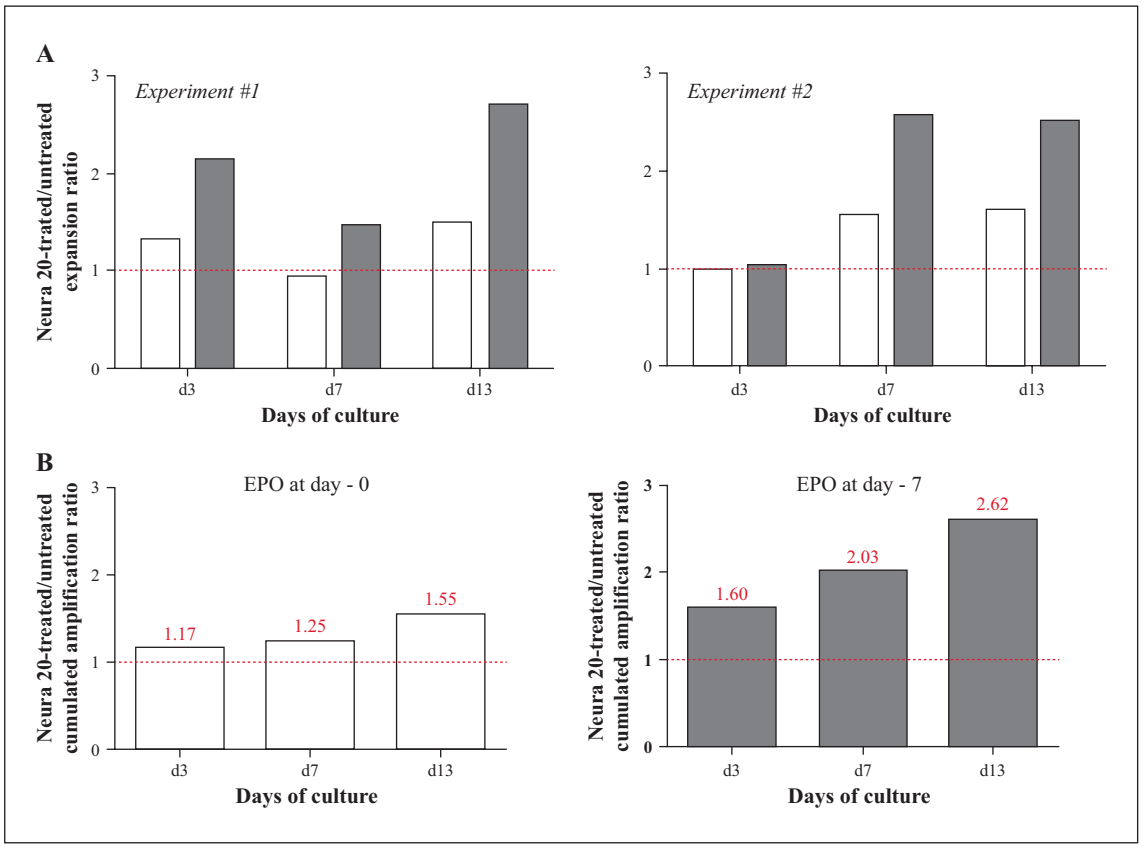

Figure 4

Expansion ratios obtained with EPO added either at day 0 or day 7. Red cell progenitors were cultured in complete liquid medium containing rhIL-3 + rhIL-6 + rhSCF. The comparative effect of EPO added either at day 0 or at day 7 was tested. The baseline, shown as a red dashed line, corresponds to the value of 1 for untreated progenitors. **(A)** Cells were counted at days 3, 7 and 13 and the comparative effect of the addition of EPO either at day 0 or at day 7 was tested. Proliferation ratios (Neura 20-treated/untreated) obtained were calculated between untreated cells and neuraminidase-treated cells (20 mU/mL) at every time interval, as a function of EPO addition time: EPO was added at day 0 (white bars) or at day 7 (grey bars). The baseline, indicated as a red dashed line, corresponds to the value of 1 for untreated progenitors. Results represented are from two representative experiments, #1 (on the left) and #2 (on the right). **(B)** Cells were counted at days 3, 7 and 13 and cumulated expansion ratios (Neura 20-treated/untreated) were calculated between untreated and neuraminidase-treated cells at every time interval as a function of rhEPO addition time: EPO was added at day 0 (white bars) or at day 7 (grey bars). Results are the mean of two separate representative experiments. Numbers on each bar indicate the value 1 corresponding to the expansion ratio for non-treated progenitors.

more generally the myeloid cell lineages, we tested the activity of 20 mU/mL of neuraminidase on the growth of granulocytic and megakaryocytic cells.

Growth of granulocytes is reduced in the presence of neuraminidase

Granulocyte progenitors were cultured under conditions as stated in the Materials & Methods section. In *figure 7A*, we show that the addition of neuraminidase led to a clear decrease in total granulocytic cell expansion at day 13 (648 ± 414-fold for untreated *versus* 264 ± 100-fold for neuraminidase-treated cells). No effect of neuraminidase on granulocyte cell expansion was observed until day 7, and the negative consequences on cell growth took place thereafter (*figure 7B*). Cell growth, starting from 1×10^5 $CD34^+$ cells at day 0, reached a mean of $6.5 \pm 4.1 \times 10^7$ for untreated *versus* $2.6 \pm 1.1 \times 10^7$ for neuraminidase-treated cells at day 13. Although neuraminidase had a deleterious effect on granulocyte progenitor amplification, the total granulocytes number obtained at the end of culture was not significantly decreased (see also *figure 8*).

Growth of megakaryocytes is not significantly altered by neuraminidase

$CD34^+$ cells were grown in the presence of the thrombopoietin receptor agonist AF13948 for 13-15 days to obtain a pure population of megakaryocytes. We obtained a 25-fold, mean cell expansion by the end of the culture. The addition of 20 mU/mL of neuraminidase did not favor cell growth and even led to a slight but non-significant drop (21-fold mean cell expansion), when compared to untreated cells (*figures 7C, D*) (see also *figure 8B*). Finally, addition of neuraminidase does not improve MK culture.

Cells expanded under neuraminidase treatment display normal phenotypes and a non-apoptotic status

We wondered whether neuraminidase could change the appearance and phenotype of the cells generated in culture. With that aim, we performed both cytological analysis and immunophenotyping of cells produced from the different culture conditions. After May-Grünwald-Giemsa coloration, we did not observe any cytological or morphological abnormalities of the

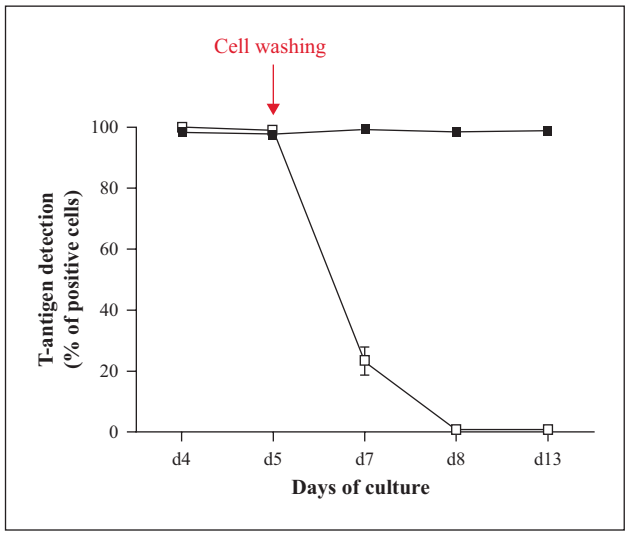

Figure 5

T-antigen positivity on cell surface and disappearance after cell washing. RCPs cultured for five days in the presence of neuraminidase (20 mU/mL) were harvested and washed (white squares) or left unwashed (black squares). Then, cells were cultured again without neuraminidase for washed cells or with neuraminidase for unwashed cells. At the indicated culture times, cells were harvested again and T-antigen detection was performed using the FITC-conjugated *Arachis hypogaea*-specific lectin. Cell washing is indicated by the red arrow. Data are represented as the percentages of positive cells (means ± SEM of three independent determinations).

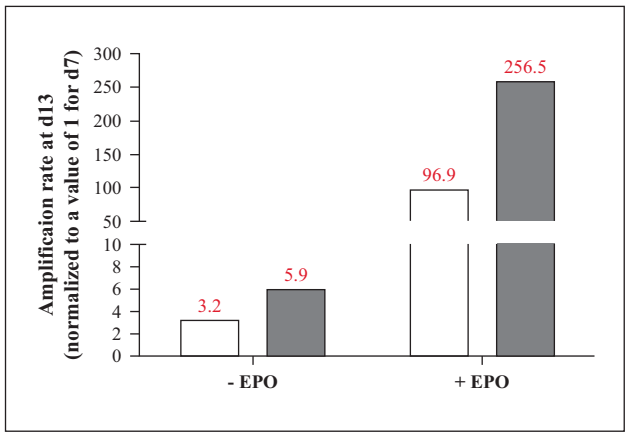

Figure 6

Amplification rates of RCPs with or without neuraminidase. Progenitors were cultured for 13 days with (dark bars) or without (white bars) neuraminidase 20 mU/mL. EPO iwas added to the culture medium (+EPO) at day 7 or not (-EPO). After 13 days, cells were counted and amplification rates determined. Results are normalized to a value of 1 for the cell number obtained at day 7 in each condition. Data shown are from a representative experiment.

cells from neuraminidase-containing cultures (data not shown). Cells of each cell lineage derived from either neuraminidase-treated or -untreated cultures were tested for specific surface markers. (i) For the red cell lineage, a similar cell phenotype evolution was observed: the kinetics of the appearance of GPA (CD235a)-positive cells after addition of EPO were not changed by the presence of neuraminidase (*figure 9A*). (ii) Concerning the granulocytic-monocytic cell lineage, the appearance of CD14 and CD15 markers was not significantly altered by the addition of neuraminidase to the cultures (*figures 9B, C*). (iii) Concerning the megakaryocytic cell lineage, the expression of CD41a was not significantly altered when tested from between day 7 to day 13 of the cultures (*figure 9D*). By contrast, the number of CD42b-positive cells was reduced in presence of neuraminidase (*figure 9E*). This is in accordance with previous demonstrations of the susceptibility of this antigen (platelet GP1b protein) to the action of neuraminidase [16]. On the whole, no increase in the percentage of apoptotic cells, as tested by Annexin V staining, was detected in the presence of neuraminidase. Additionally, there was no increase in the number of dead cells as demonstrated with the trypan blue exclusion count (data not shown).

Clonogenic progenitors are not affected by the presence of neuraminidase

To determine whether neuraminidase could influence the number and/or the quality of CFCs, cells from liquid cultures were plated in semi-solid culture conditions with a mixture of cytokines as described in the Materials & Methods section. Colonies were counted and categorized after 10-15 days of culture (depending on the duration of the liquid cultures before plating). *Table 1* shows that the number or type of colonies of both RCPs and total myeloid progenitors (calculated ratio) was not significantly modified by neuraminidase (see also *table 2* for the absolute number of colonies counted).

DISCUSSION

We show here that the addition of neuraminidase to cultures of $CD34^+$ cells can lead to improved EP production. Such enhancement of cell expansion is restricted to the erythroid cell lineage: actually, under our culture conditions, growth of MK cells remained unaffected by neuraminidase, whereas the development of granulocytes is clearly impaired. Expanded cells from the red cell lineage obtained in this manner displayed a normal phenotype that could not be distinguished from untreated cells, either by surface markers analysis or by morphological examination. Expression of the T-antigen on the cell surface, due to desialylation of membrane proteins by neuraminidase, was fully reversible, and thus represents a control for the safety of such treatment of RBC precursors. Nevertheless, the complete safety of such treatment remains to be proven in an *in vivo* setting.

We have demonstrated that red cell growth remains dependent upon the presence of EPO, suggesting that desialylation of the EPO receptor does not render the cultured red cell progenitors EPO-autonomous. Besides, the fact that the addition of rhEPO at day 0 (thus triggering EPs to differentiate early) resulted in a significant reduction in EP expansion suggested that the initial commitment of $CD34^+$ cells to erythroid differentiation renders them partially refractory to the expansive effect of neuraminidase. Likewise, it must be emphasized that, as far as cultures of megakaryocyte and granulocyte progenitors are concerned, either TPO agonist or G-CSF respectively were added at the beginning of cultures (day 0), leading these cells to initiate their commitment to differentiation. Therefore, we assume that initial differentiation triggering of progenitors leads to the suppression of neuraminidase-induced expansion potential. The sialomucin CD164 is present on human hematopoietic progenitors and is a potent

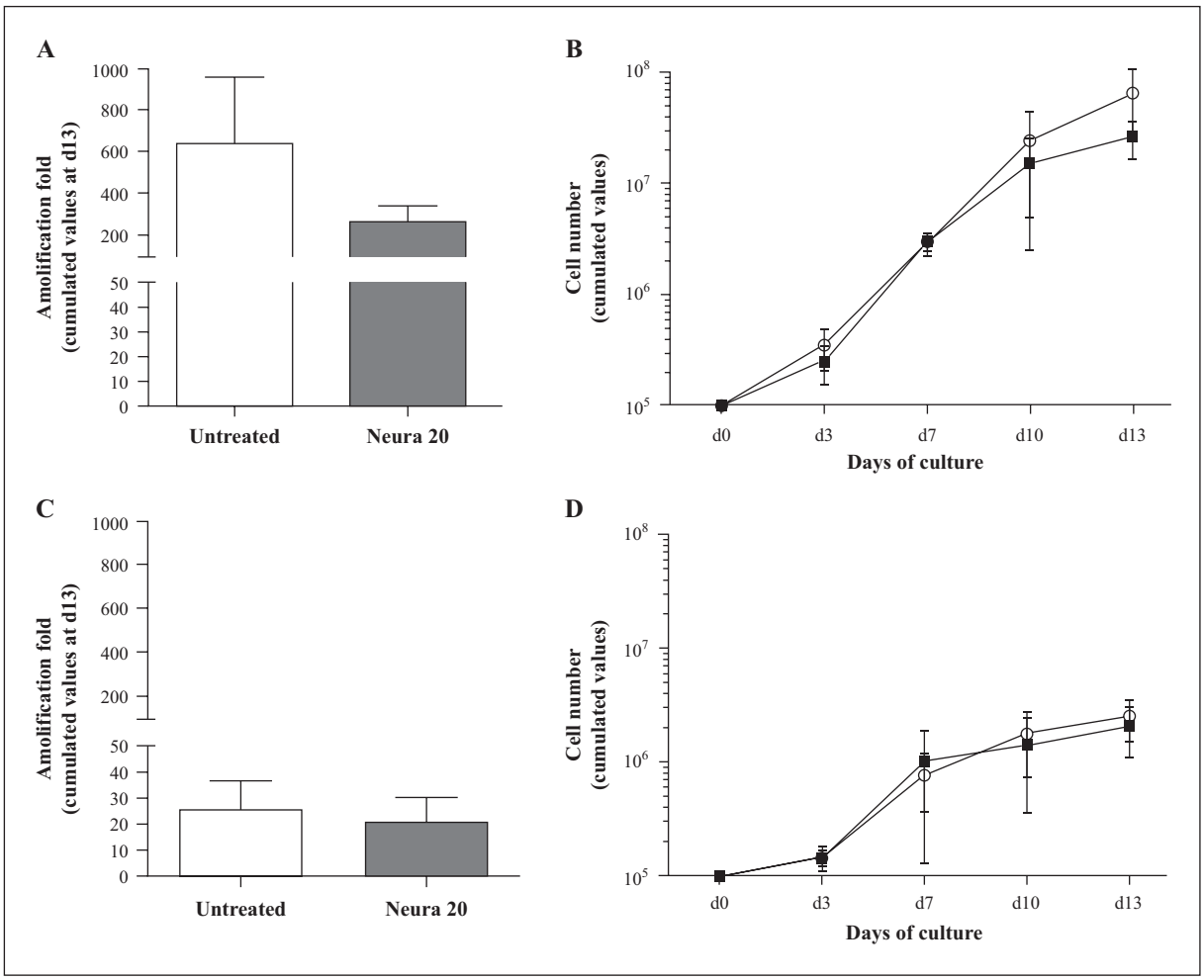

Figure 7

Expansion of CD34⁺ progenitors directed to granulocyte **(A, B)** or to megakaryocyte differentiations **(C, D)**. Cultured progenitors were treated with 20 mU/mL of neuraminidase (black bars) or not (white bars). **(A, C)**: cell amplification folds. At day 13, cells were counted and expansion folds (cumulated values between day 0 and day 13) were determined (means of 3 independent experiments). **(B, D)**: cell numbers generated in the cultures from day 0 to day 13. The cumulated numbers of cells were determined during the culture of untreated (white circles) or 20 mU/mL neuraminidase-treated (black squares) progenitors between day 0 and day 7, if 100,000 cells were seeded at day 0. Data are represented as the mean ± SEM of 3 independent experiments.

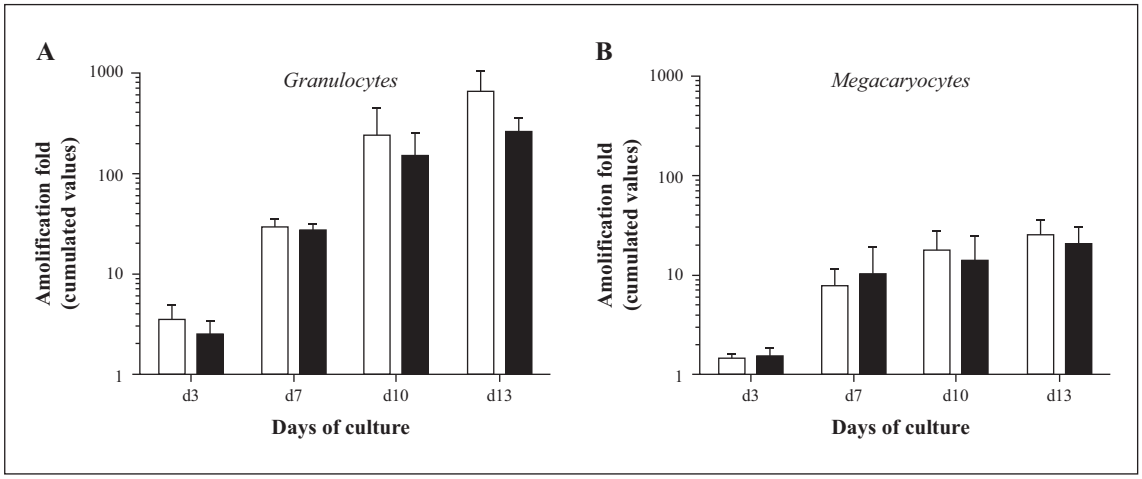

Figure 8

Expansion of CD34⁺ progenitors either directed to granulocyte differentiation or to megakaryocyte differentiation.
Cultured red cell progenitors were treated with 20 mU/mL of neuraminidase (black bars) or not (white bars). Cells were counted at day 3, day 7, day 10 and day 13 cells to determine the cumulated numbers of cells during the culture. These results allowed calculating cumulative expansion folds during the culture: these results are shown as histograms. Data are represented as the mean ± SEM of three independent experiments. **(A)** Cumulated amplification-fold data obtained for granulocytes cultivated with G-CSF. **(B)** Cumulated amplification-fold data obtained for megakaryocytes cultivated with TPO receptor agonist.

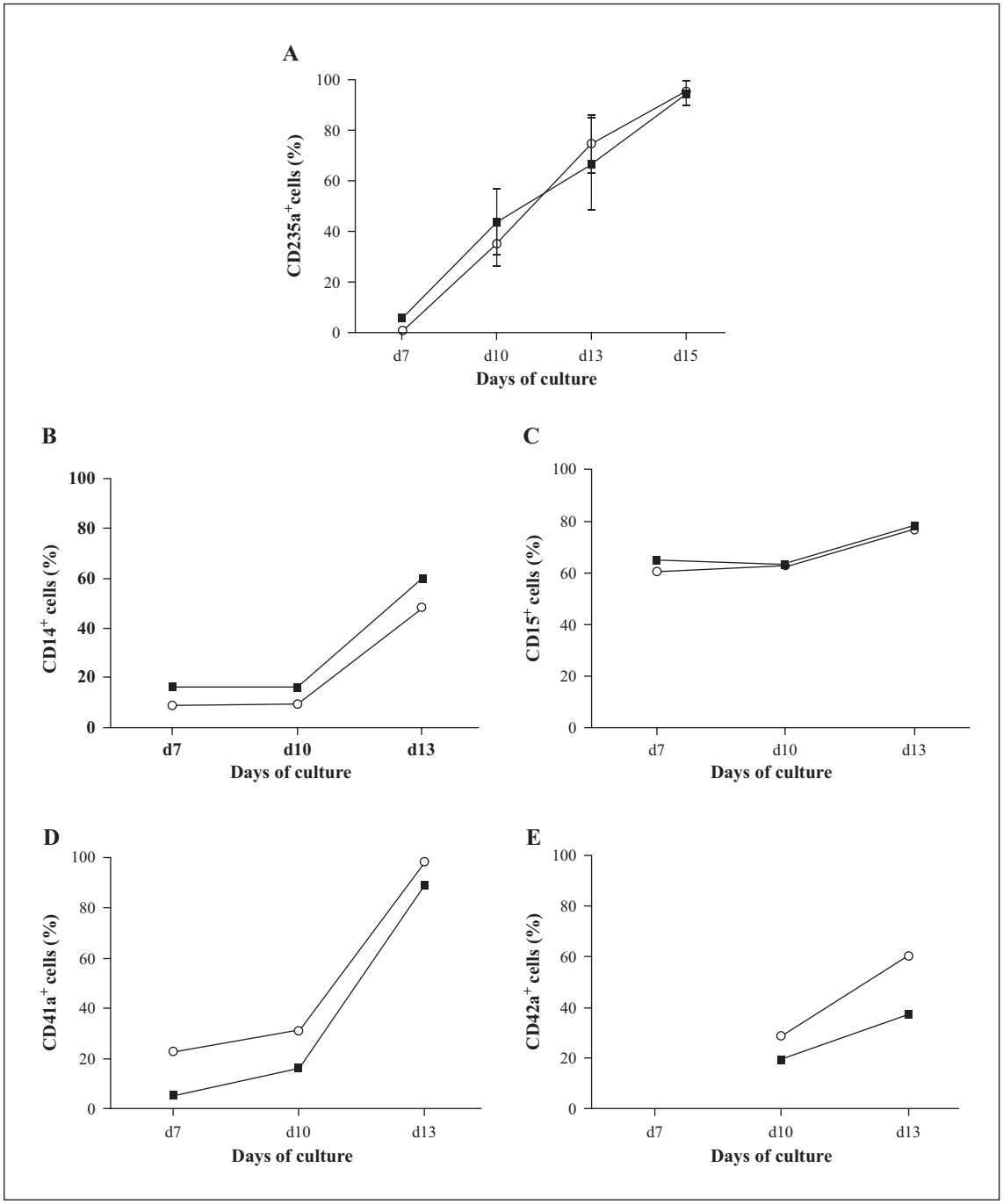

Figure 9

Phenotypic analysis of the cells during the culture.
$CD34^+$ cells cultured in the absence (white circles) or presence (black squares) of neuraminidase 20 mU/mL were directed to red cell differentiation in the presence of rhEPO **(A)**, to granulocyte differentiation in the presence of G-CSF **(B, C)** or to megakaryocyte differentiation in the presence of TPO receptor agonist **(D, E)**. At different times of the culture, during expansion and differentiation, phenotypic markers of the cells were analyzed by flow cytometry for the expression of (i) CD235a (glycophorin A) for red cell differentiation, (ii) CD14 and CD15 for granulocyte differentiation, or (iii) CD41a and CD42b for megakaryocyte differentiation. Results in (A), (B), (C), (D) and (E) are percentages of positive cells (means ± SEM) obtained from 2-5 independent determinations.

negative regulator of hematopoiesis [17]. We addressed whether the treatment of hematopoietic cell cultures by neuraminidase in culture could lead to the disappearance or reduction of CD164 on the cell surface. Two experiments were performed and both led us to the conclusion that there was no decrease in CD164 in the presence of neuraminidase (data not shown).

Previous papers have reported contradictory results concerning the action of neuraminidase on the regulation of erythropoiesis in human or mouse models: either increases [18, 19] and decreases [20-22] in RBC progenitors after treatment with neuraminidase have been described. It must be emphasized that these teams used cell models and culture conditions very different from ours, and thus comparisons with our results would be largely irrelevant and unreliable. We tried to better understand the mechanisms of action of the improvement of red cell expansion by neuraminidase. With that aim, several routes were explored using specific inhibitors: Ly294002 for the PI3 kinase pathway, rapamycin for the mTOR pathway, UO126 for the MAP kinase pathway, and imatimib as inhibitor of c-abl and c-kit. In all cases, no decrease in

Table 1
CFC potential ratios.

	Day 1	Day 3	Day 6
Red cell colonies ratio	1.10	1.17 ± 0.1	1.04 ± 0.3
Total colonies ratio	1.04	1.02 ± 0.2	0.76 ± 0.2

Ratios are calculated for the number of methylcellulose colonies obtained from neuraminidase 20 mU/mL-treated *versus* untreated cells. Red cell progenitor colonies counted (duplicate determinations) are: BFU-E + CFU-E; total progenitor cell colonies are: BFU-E + CFU-E + CFU-GM + CFU-G + CFU-M. Days represent the expansion periods of culture in liquid medium, with or without neuraminidase, before plating in semi-solid conditions.

Table 2
CFC potential results obtained for day 3 and day 6 (number of colonies).

Days	Day 3	Day 6
Nbr of red cells colonies		
Untreated	119.3 ± 45.1	38.3 ± 8.9
Neura 20	145 ± 68	37.67 ± 6.9
Nbr of colonies (total)		
Untreated	210.3 ± 59.8	85 ± 22
Neura 20	218.7 ± 87.6	58.67 ± 2.4

Number of methylcellulose colonies obtained from neuraminidase 20 mU/mL-treated cells or from untreated cells. Red cell progenitor colonies counted are: BFU-E + CFU-E; total progenitor cell colonies are: BFU-E + CFU-E + CFU-GM + CFU-G + CFU-M. Data represented are means ± SEM (n = 3). Days represent the expansion periods of the culture in liquid medium, with or without neuraminidase, before plating in semi-solid conditions. Duplicated determinations are made for each condition.

neuraminidase-mediated EP cell expansion was observed when compared to cell expansion in the absence of inhibitor (data not shown). Further investigations are needed to clarify the activity of neuraminidase on erythroid cell progenitors.

Previously, we had shown [13] that our double-step culture model generated normal red cells (with a normal phenotype and morphology), with 97 % of cells belonging to the erythrocytic lineage and very faint contamination by other myeloid cell lineages. In that study, we focused on the proliferation of red cell lineage progenitors: however, the normal differentiation fate of these cells was also explored and confirmed through generation of BFU-E and CFU-E, in semi-solid culture tests. The correct morphology of further *in vitro*-generated red cell precursors was also confirmed under microscopic examination (data not shown).

In vitro proliferation and expansion of erythroid progenitors represent an important achievement as a precursor to obtaining high numbers of mature RBCs for a transfusion context. Actually, it has been shown that *in vitro*-generated red cells from $CD34^+$ hematopoietic stem cells or from human induced pluripotent stem (iPS) cells were able to persist in the circulation for several weeks after *in vivo* injection into immuno-deficient mice [23, 24]. It would be challenging to determine whether the addition of neuraminidase is able to improve the generation of erythroid progenitors from iPS cells. Furthermore, *ex vivo* expansion of erythroid cells represents a useful tool to better study the fundamental regulation mechanisms of these cells and to study the activity of certain pathogens that induce erythroid cell lysis, such as the Parvovirus B19 [25] or *Plasmodium falciparum* [26].

Moreover, other culture conditions can also lead to RBC expansion. For example, it is well established that cultures in hypoxic conditions result in improved cell expansion, particularly for red cell precursors, thus providing a tool to optimize the technology of *ex vivo* production of red blood cells. It will be of interest to determine whether enhanced expansion of neuraminidase-treated red cell progenitors is improved futher by hypoxia.

In conclusion, we show here that *in vitro* treatment of $CD34^+$ cells with neuraminidase considerably improves red cell progenitor expansion under specific culture conditions. This effect could be used to enhance red cell production *in vivo*. Finally, such an RBC expansion model in stromal-free and serum-free culture conditions provides a safe background as regards both basic research and transfusion use.

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