Concentrative Uptake of Cyclic ADP-ribose Generated by BST-1+ Stroma Stimulates Proliferation of Human Hematopoietic Progenitors*

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Cyclic ADP-ribose (cADPR) is an intracellular calcium mobilizer generated from NAD+ by the ADP-ribosyl cyclases CD38 and BST-1. cADPR, both exogenously added and paracrinically produced by a CD38+ feeder layer, has recently been demonstrated to stimulate the in vitro proliferation of human hematopoietic progenitors (HP) and also the in vivo expansion of hematopoietic stem cells. The low density of BST-1 expression on bone marrow (BM) stromal cells and the low specific activity of the enzyme made it unclear whether cADPR generation by a BST-1+ stroma could stimulate HP proliferation in the BM microenvironment. We developed and characterized two BST-1+ stromal cell lines, expressing an ectocellular cyclase activity similar to that of BST-1+ human mesenchymal stem cells, the precursors of BM stromal cells. Long term co-culture of cord blood-derived HP over these BST-1+ feeders determined their expansion. Influx of paracrinically generated cADPR into clonogenic HP was mediated by a concentrative, nitrobenzylthioinosine- and dipyrindamole-inhibitable nucleoside transporter, this providing a possible explanation to the effectiveness of the hormone-like concentrations of the cyclic nucleotide measured in the medium conditioned by BST-1+ feeders. These results suggest that the BST-1-catalyzed generation of extracellular cADPR, followed by the concentrative uptake of the cyclic nucleotide by HP, may be physiologically relevant in normal hemopoiesis.

The self-renewal capacity of hematopoietic progenitors (HP) in the bone marrow (BM) is a fundamental process in the physiology of hemopoiesis. The role of the BM stroma in providing HP with soluble factors essential to their proliferation and differentiation is well established (1, 2). However, the nature of the signals and the mechanisms by which stromal cells regulate the behavior of HP remain largely to be defined. Particularly, it is unknown whether perturbations of the balance between growth-stimulatory and -inhibitory factors may influence the expansion and self-renewal capacity of the hemopoietic reservoir.

Recently, we have demonstrated that the potent intracellular calcium mobilizer cyclic ADP-ribose (cADPR) (3, 4) features properties of a novel hemopoietic growth factor (5, 6). Co-infusion of human HP with murine stromal cells transfected with the human ectocellular ADP-ribose cyclase CD38 improves hemopoietic stem cells engraftment into NOD/SCID mice (7). The paracrine interaction between stroma and HP has been investigated in a transwell co-culture setting where human HP were cultured over confluent monolayers of murine stromal cells; NAD-ase, NAD+ glycohydrolase; wt, wild-type.

1 The abbreviations used are: HP, hematopoietic progenitors; BM, bone marrow; CADR, cyclic ADP-ribose; NT, nucleoside transporter; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; BM-NT, nitrobenzylthioinosine; [Ca2+]i, intracellular calcium concentration; LTC, long term culture; LTC-IC, LTC-initiating cells; CB, cord blood; CB MNC, cord blood-derived mononuclear cells; CFC, colony forming cells; IFN-γ, interferon-γ; MSC, mesenchymal stem cells; NAD-ase, NAD+ glycohydrolase; wt, wild-type.
The fact that native hematopoietic stroma expresses the ADP-ribosyl cyclase BST-1 instead of CD38 may be advantageous in the BM microenvironment; CD38 is a transmembrane, oligomeric, catalytically active transporter of cADPR (12), while BST-1 is a glycosylphosphatidylinositol-anchored ectoenzyme, possibly incapable of cADPR transport, and with a significantly lower specific activity compared with CD38 (13–17). Thus, while co-expression of CD38 with connexin 43 (as occurs in the transduced stromal cell lines) results in a significant (2–3-fold) increase of the \([\text{Ca}^{2+}]_{i}\), of CD38+ stromal cells (18), the same may not hold true for BST-1. Accordingly, expression of BST-1 on the hematopoietic stroma might represent the critical feature ensuring paracrine production of HP-expanding cADPR, while limiting the extent of the \([\text{Ca}^{2+}]_{i}\) perturbation induced on stromal cells by autocrine cADPR.

The aims of this study were: (i) to compare the effect of LTC over CD38\(^{+}\) versus BST-1\(^{+}\) stroma on the in vitro clonogenic capacity of human HP and (ii) to investigate the mechanism of uptake of paracrinally produced cADPR by human HP.

Two BST-1-expressing stromal cell lines were generated and their biochemical characterization demonstrated distinctive properties as compared with the corresponding CD38-expressing cells. LTC of cord blood-derived HP over these BST-1+ feeders significantly increased colony output compared with controls, co-cultured over cyclase negative feeders, in sharp contrast with the inhibitory effect induced by LTC over CD38+ stroma. A concentric transport of paracrinally generated cADPR into HP was found to be mediated by a NBMPR- and dipyridamole-sensitive CNT. These results are consistent with a role of BST-1-generated cADPR in the expansion of human HP in the bone marrow microenvironment.

**EXPERIMENTAL PROCEDURES**

**Samples**—Cord blood (CB) samples were obtained from umbilical and placental tissues scheduled for discard. CB-derived mononuclear cells (CB MNC) were isolated by centrifugation of the blood on Ficoll Paque Plus (Amersham Bioscience). CD34+ cells (the MNC enriched in HP) were separated by immunomagnetness on magnetic columns (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer’s instructions, as described (5). Human-derived cells were maintained in glutamine-containing Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), in a humidified 5% CO2 atmosphere at 37 °C.

**Transfection of 3T3 Cells with BST-1**—The complete coding sequence of the murine full-length BST-1 cDNA was recovered as EcoR1 fragment (from plasmid pBST-1 kindly provided by Dr. Toshiro Hirano, Osaka, Japan; Ref. 17) and cloned in the same restriction site of the pcDNA1.1 expression plasmid. The plasmid obtained was sequenced on both strands of the cDNA to verify the presence of the correct insert. The pcDNA3.1/BST-1 construct was transfected into 3T3 cells with Lipofectamine Plus (Invitrogen, Milan, Italy), according to manufacturer’s instructions. One day after gene transfer, Genetin (1 mg/ml) was added to the culture medium, and transfected cells were cultured in antibiotic selection.

Five magnetic bead immuno-selections for expression of BST-1 were performed on transfected cells using anti-BST-1 (CD157) antibody (Sigma, Milan, Italy), as described (5). Expression of BST-1 was monitored by assay of the ectacellular ADP-ribosyl cyclase activity (see below).

**FACS Selection of COS-7 Cells for BST-1 Expression**—The simian epithelial cells COS-7 naturally express BST-1 (see “Results”). Two cell populations, expressing low (BST-1dim) and high (BST-1bright) levels of BST-1 were selected from wild-type COS (COS-1) by repeated FACS sorting, using a commercial anti-human BST-1 (CD157) antibody (see above) and a FITC-labeled anti-mouse IgG antibody (Sigma, Milan, Italy). Both monoclonal antibodies were applied under previously determined saturating conditions using the Indirect Immunofluorescence assay. Briefly, 2 μl of anti-BST-1 monoclonal antibody were added to 1 × 10⁶ COS-7 cells in 100 μl of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (PBS-BSA) and cells were incubated for 30 min on ice, in the dark. Cells were then washed twice in PBS, and 150 μl of the second monoclonal antibody (at a 1:75 dilution in PBS-BSA) was then added and cells were further incubated for 30 min. Finally, cells were washed twice and resuspended in PBS at a concentration of 1 × 10⁶ cells/ml. Deoxyribonuclease I (from bovine pancreas, Calbiochem Biochemicals, Nottingham, UK) and propidium iodide at 1 μg/ml (Sigma Aldrich Fine Chemicals, Milan, Italy) were added to the cells, which were then filtered on a 70-μm cell strainer (Falcon-BD) immediately before sorting. Control cells were exposed only to the secondary antibody.

Flow cytometric analysis and cell sorting were performed by using a FACS Vantage SE flow sorter (BD Biosciences Immunocytometry Systems, Palo Alto, CA) equipped with an air-cooled 50-megawatt argon ion laser. BST-1+ COS-7 cells were gated within a viable, propidium iodide-negative, population using a Forward Scatter versus FL-3 logarithmic amplified channel dotplot. In each experiment, sorting gates were set to sort both the dimmest and the brightest BST-1+ populations in the same two-way sorting experiment. A total of four sorting experiments were performed: between each round, the COSdim and COSbright populations were expanded in vitro.

**Assay of ADP-ribosyl Cyclase Activity**—Ectocellular ADP-ribosyl cyclase activity was measured by incubating 3 × 10⁶ cells in a total volume of 400 μl of PBS-glucose (10 mM) with 0.1 mM NAD⁺. At different times (0, 1, and 4 h), 100-μl aliquots were withdrawn. Reactions were stopped by addition of 220 μl of 0.9 M perchloric acid to each aliquot. After deproteinization, acid was removed and cADPR content was measured in each aliquot according to the enzymatic cycling assay (20). Protein determination was performed on an aliquot of the incubation (21).

**Determination of the Intracellular Concentration of NAD⁺ and cADPR**—BST-1+ and control 3T3 cells (5 × 10⁵) were lysed at 4 °C in 0.5 ml of 0.6 M perchloric acid. An aliquot was diluted 200-fold in PBS to NAD⁺ levels determination. This remaining volume was subjected to deproteinization, acid extraction, and NAD⁺ degradation, and cADPR content was determined by the enzymatic cycling assay (20). Intracellular NAD⁺ and cADPR content was expressed as picomole/mg of protein.

**Determination of the [Ca²⁺]i**—Basal intracellular free calcium concentration ([Ca²⁺]i) was determined as described (19) on FURA 2AM-loaded 3T3 fibroblasts and COS cells. For determination of the [Ca²⁺]i, on CD34+ cells, aliquots of 2 × 10⁵ cells were incubated in a total volume of 1.0 ml, in the presence of 50 μM cADPR, without or with 10 nM NBMPR. After 24 h, 10 μM FURA 2-AM was added, and cells were further incubated for 60 min. Thereafter, cells were washed twice by centrifugation, resuspended in 0.3 mM of glucose-supplemented standard saline (5), and calcium measurements were performed at room temperature in a 0.5-ml cuvette. The basal [Ca²⁺]i was determined as described previously (19).

**Co-culture Experiments**—All cell lines used as feeder layers were cultured in Dulbecco’s modified Eagle’s medium, as described (19). Transfected cells were maintained under Genetin (1 mg/ml) selection in the same medium. After irradiation (1500 Gy), feeder cells were seeded into 24-well plates in complete medium, without Genetin, and allowed to grow to confluence. Cells from cultures that grew into the feeder layer were seeded in transwells (Costar, Milan, Italy) at 2 × 10⁵/well in LTC medium (Myelocult, Stem Cell Technologies, Vancouver, Canada) or irradiated, confluent (≈2 × 10⁶ cells) feeder layers. At weekly intervals, transwells were transferred over freshly irradiated feeders, cells were recovered from individual wells and counted, and aliquots were seeded in growth factor-supplemented semisolid medium to determine the content in clonogenic precursors (see below).

**Semisolid Colony Growth Assay**—After liquid culture, aliquots of 2 × 10⁴ MNC were seeded in growth factors-supplemented methylcellulose medium (Methocult, Stem Cell Technologies, Vancouver, British Columbia, Canada) and cultured at 37 °C in a humidified, 5% CO₂ atmosphere, as described (5). After 14 days, colonies were scored and identified according to standard criteria. Colonies grown from cells preincubated for 1–7 days in liquid culture in the absence of added cytokine identify the committed HP, i.e. the colony-forming cells (CFC) (22). Conversely, colonies that develop from cells after 5 weeks of liquid culture identify the uncommitted HP, i.e. long term culture-initiating cells (LTC-IC) (23).

**RESULTS**

**Development and Biochemical Characterization of BST-1+ Stromal Cell Lines**—The fact that native hematopoietic stroma
expresses BST-1 instead of CD38 prompted us to develop two different BST-1<sup>+</sup> stromal cell lines, to be used as feeders in co-culture experiments, aimed at establishing the effect of LTC over BST-1<sup>+</sup> stroma on HP growth. Murine 3T3 fibroblasts were transfected with the sense cDNA for murine BST-1, while simian COS-7 epithelial cells, which constitutively express BST-1 (Table I), were sorted into high (COS<sup>bright</sup>) and low (COS<sup>dim</sup>) expressing BST-1<sup>+</sup> subpopulations by FACS, using a commercially available monoclonal antibody against human BST-1 (see “Experimental Procedures”).

Stable levels of BST-1 expression, as detected by enzymatic analysis, were obtained on 3T3 cells after five cycles of immunomagnetic selection. A clear-cut separation of BST-1<sup>dim</sup> and BST-1<sup>bright</sup> COS subpopulations was observed after four cycles of FACS selection (Fig. 1) and routinely checked thereafter, during the course of the co-culture experiments.

The levels of ecto-cyclase activity measured on the BST-1<sup>+</sup> cell lines were similar (1.65 <i>versus</i> 1.72 pmol cADPR/min/mg for BST-1<sup>+</sup> 3T3 and COS<sup>bright</sup>, respectively) and comparable with the cyclase activity constitutively expressed by BST-1<sup>+</sup> human mesenchymal cells, the progenitors of BM stroma, expanded in vitro from BM aspirates (24, 25) (1.65 ± 0.38 pmol cADPR/min/mg). On the other hand, CD38<sup>+</sup> 3T3 fibroblasts expressed ~50 times higher cyclase activity (91 pmol cADPR/min/mg), in line with the higher specific activity reported for CD38 as compared with BST-1 (13–17). The intracellular cADPR concentration ([cADPR]<sub>i</sub>) in CD38<sup>+</sup> 3T3 cells was accordingly 1 log higher than that measured in BST-1<sup>+</sup> 3T3 and in COS<sup>bright</sup> cells (13 and 7 times higher, respectively) (Table I). The limited increase of the [cADPR]<sub>i</sub> of BST-1<sup>+</sup> cells was not paralleled by any significant increase of the basal [Ca<sup>2+</sup>]<sub>i</sub>, compared with the respective controls (3T3 cells transfected with the empty vector and unsorted COS cells, respectively) (Table I). On the contrary, expression of CD38 in 3T3 fibroblasts reduced the [NAD+ NADH]<sub>i</sub> to ~50% of control values (Table I), similarly to earlier results (19).

The cADPR concentration in the medium conditioned for 3 days by CD38<sup>+</sup> 3T3 was found to be in the subnanomolar range (0.51 ± 0.14 nM, n = 6), as detected by enzymatic cycling assay (20). In the medium conditioned by BST-1<sup>+</sup> 3T3 the [cADPR]<sub>i</sub> was even lower (0.18 ± 0.09 nM, n = 5) and near the sensitivity threshold of the assay.

**Table I**

| ADP-ribo cyclase | [cADPR]<sub>i</sub> | [NAD+ NADH]<sub>i</sub> | [Ca<sup>2+</sup>]<sub>i</sub> |
|-----------------|-----------------|-----------------|-----------------|
| 3T3 control     | ND              | 5.11 ± 0.8      | 25 ± 7          |
| 3T3 BST-1<sup>+</sup> | 0.32 ± 0.06     | 6.1 ± 0.8       | 23 ± 5          |
| 3T3 CD38<sup>+</sup> | 3.75 ± 0.6      | 3.32 ± 0.2      | 45 ± 8          |
| COS<sup>dim</sup> | 0.9 ± 0.02      | 4.3 ± 0.5       | 21 ± 3          |
| COS<sup>bright</sup> | 2.5 ± 0.8       | 4.2 ± 0.7       | 22 ± 3          |
| 3T3 BST-1<sup>+</sup> | 1.72 ± 0.5      | 4.8 ± 0.7       | 24 ± 2          |

<sup>a</sup> Not detectable.

**Fig. 1.** Staining patterns for BST-1 antigen analyzed by flow cytometry. A, COS-7 wild-type population histogram plot. B, overlaid histogram plots of sorted Dim (left histogram) and bright (right histogram) COS-7 populations after four rounds of sorting. For further details, see “Experimental Procedures.” Results from a representative experiment are shown.

Previous observations had shown an increased, [Ca<sup>2+</sup>]<sub>i</sub>-related IFN-γ concentration in the medium conditioned by CD38<sup>+</sup> 3T3 feeders compared with controls (6); this prompted us to measure the [IFN-γ] in the medium of confluent monolayers of BST-1<sup>+</sup> 3T3 and of COS<sup>bright</sup> cells. No significant differences were observed between these cell lines and the corresponding controls (3T3 transfected with the empty vector and COS<sup>bright</sup>), as measured with a colorimetric immunoassay (R&D Systems, Minneapolis, MN) on 7-day medium from confluent, irradiated feeder layers (6) (data not shown).

**Long Term Culture of CB MNC over BST-1<sup>+</sup> Stroma—**Long term co-culture in transwells of CB MNC over undiluted, CD38-transfected 3T3 fibroblasts proved to remarkably decrease their clonogenic activity. This effect was attributed to the autocrine cADPR-[Ca<sup>2+</sup>]<sub>i</sub>-loop in the CD38<sup>+</sup> 3T3, which induced overproduction of hemopoiesis-inhibiting IFN-γ (6). To compare the effects of stromal BST-1 and CD38 on HP expansion, CB MNC were co-cultured in transwells over either BST-1<sup>+</sup> or CD38<sup>+</sup> 3T3 feeders and their clonogenic capacity was assayed weekly for up to 5 weeks (see “Experimental Procedures”). Controls were cultured over 3T3 fibroblasts transfected with the empty vector pcDNA 3.1.

As shown in Table II, the clonogenic activity, measured both as CFC frequency and total colony number, was significantly higher in CB MNC cultured over BST-1<sup>+</sup> feeders compared with controls, starting from the 2nd week of culture. Con-
versely, co-culture over CD38\textsuperscript{−} feeders strongly inhibited colony production from the 1st week of culture (Table II). Colony output of CB MNC cultured over CD38\textsuperscript{−} cells transfected with antisense CD38 was not significantly different from controls (data not shown). In previous experiments, addition of NAD\textsuperscript{+} glycohydrolase (NAD-ase) to the medium prevented the stimulatory effect of a short term co-culture over CD38\textsuperscript{−} stroma on CFC output (6). This result was taken as an indication that release of NAD\textsuperscript{+} from the feeder cells provides the substrate for the extracellular generation of cADPR. Here we tested the effect of NAD-ase on the long term co-culture of CB MNC over BST-1\textsuperscript{+} 3T3. Addition of NAD-ase (2 units/ml, twice weekly for 2 weeks) to the culture medium prevented the stimulation of colony output by the BST-1\textsuperscript{+} feeder: colony frequencies (CFC/10\textsuperscript{5} MNC) were 22\textpm{}5 versus 7 \textpm{}1 for BST-1\textsuperscript{+} and control feeder, respectively, and 7 \textpm{}2 versus 6 \textpm{}1 for BST-1\textsuperscript{+} and control feeder in the presence of NAD-ase (mean \textpm{} S.D. from three experiments).

The effect of long term co-culture over a BST-1\textsuperscript{−} feeder was also investigated with the different COS feeders, COS\textsuperscript{wt} (controls), COS\textsuperscript{bright} and COS\textsuperscript{dim}, expressing distinctively different levels of ectocyclase activity (Table I). COS\textsuperscript{bright} feeders showed an improved long term supporting capacity over controls; colony output was higher from the 3rd week of co-culture compared with COS\textsuperscript{wt}. Conversely, the cloning capacity of CB MNC cultured over COS\textsuperscript{dim} feeders was markedly reduced compared with controls, again from the 3rd week onwards (Table IIIA). These results were confirmed by analysis of the cell expansion factor, calculated as the ratio between the number of cells seeded in semisolid medium and the number of cells harvested from the grown colonies (Table IIIB).

\textbf{Nucleoside Transport Inhibitors Prevent the Stimulatory Effect of cADPR on Clonogenic HP—}In 3T3 fibroblasts and in Me\textsubscript{6}SO-differentiated HL-60 cells (a human promyelocytic leukemia cell line), influx of extracellular cADPR is mediated by nucleoside transporters (NT), both equilibrative and concentrative (ENT and CNT, respectively) (9, 10). To establish whether these transporters are responsible for cADPR entry into human HP, CB MNC, i.e. the cells containing a significant fraction of HP, were exposed to either of the NT inhibitors dipyridamole and nitrobenzylthioinosine (NBMPR) prior to incubation with exogenously added cADPR, the schedule of cADPR treatment of HP, CB MNC, these transporters are responsible for cADPR entry into human HP, CB MNC, or with the empty vector pcDNA3.1 (control). At weekly intervals, different numbers of cells were seeded in semisolid medium for 2 weeks, and colony growth was recorded as described under "Experimental Procedures." Results from five different experiments are shown. Each number represents the mean between at least two values, obtained by seeding different cell numbers. Statistically significant differences between the three groups are highlighted by bold numbers.

### Table II

| CFC/10\textsuperscript{5} MNC | Total CFC |
|-----------------------------|----------|
| BST-1\textsuperscript{+} | Control | CD38\textsuperscript{−} | BST-1\textsuperscript{+} | Control | CD38\textsuperscript{−} |
| 1st week | 76 | 60 | 32 | 488 | 444 | 71 |
| 2nd week | 28 | 5 | 1 | 196 | 25 | 3 |
| 3rd week | 78 | 13 | 4 | 1400 | 26 | 8 |
| 4th week | 11 | 0 | 0 | 44 | 0 | 0 |

### Table III

| A. Weeks of co-culture |
|------------------------|
| COS\textsuperscript{wt} | 2400 | 2200 | 1540 | 280 | 0 |
| COS\textsuperscript{dim} | 1900 | 2100 | 1280 | 0 | 0 |
| COS\textsuperscript{bright} | 2500 | 2640 | 2420 | 770 | 250 |

| B. Weeks of co-culture |
|------------------------|
| COS\textsuperscript{wt} | 390 | 480 | 320 | 170 | 0 |
| COS\textsuperscript{dim} | 320 | 510 | 8 | 164 | 0 |
| COS\textsuperscript{bright} | 1770 | 720 | 590 | 520 | 9 |
Thereafter, cADPR (100 μM) was added, and the cells were cultured for 24 h. Aliquots of cells were then seeded in semisolid medium for 14 days, as described under “Experimental Procedures,” for estimation of colony growth.

Colony output was increased by 100 μM cADPR (2200 ± 190 CFC/10^6 MNC) compared with untreated, control cultures (1000 ± 280 CFC/10^6 MNC; p < 0.04), and both NT inhibitors prevented the stimulatory effect of cADPR (800 ± 200 and 1,100 ± 230 CFC/10^6 MNC for 100 nM dipyridamole and 10 nM NBMPR, respectively; mean ± S.D. from four experiments). These results indicated involvement of a dipyridamole, and NBMPR-sensitive NT, in the mechanism of entry of cADPR into the clonogenic HP (CFC); however, sensitivity to both NT inhibitors is a property shared by the equilibrative NT ENT-1 (27, 28).

To investigate the possible role of concentrative NTs in mediating influx of cADPR into CFC, the effect of dipyridamole and NBMPR was assayed on a co-culture system, where CB MNC were overlaid in a transwell mode on stromal feeders, engineered to produce extracellular cADPR, i.e. 3T3 fibroblasts transfected with human CD38, sense (CD38^+/− 3T3), and antisense (CD38^− 3T3) as negative control (19). The cADPR concentration in the medium conditioned by CD38^− 3T3 was found to be in the subnanomolar range (see above), i.e. several orders of magnitude below the known Km of the equilibrative NT for cADPR, ENT2 (9, 10); this rules out any role of ENT2 in the influx of cADPR into the CFC, in close agreement with data obtained with Me₃SO-differentiated HL-60 cells (10). After 24-h co-culture of CB MNC over CD38^+/− 3T3 in the presence or absence of either dipyridamole or NBMPR, cells were seeded in semisolid medium to measure colony growth. As shown in Fig. 2, CFC output was ~2-fold higher for cells cultured for 24 h over CD38^− 3T3 feeders compared with controls, grown over CD38^+/− 3T3 (1,600 ± 200 CFC/10^6 MNC versus 900 ± 150, mean ± S.D. from five experiments; p < 0.04). The presence of dipyridamole or NBMPR completely prevented the stimulatory effect of the co-culture over CD38^− 3T3 (Fig. 2). Similar results were obtained using BST-1^+ 3T3 as feeder; CB MNC were co-cultured for 2 weeks over BST-1^+ or BST-1^− (control) 3T3, in the presence or absence of 10 nM NBMPR (added twice weekly) and then seeded in semisolid medium for colony growth. CFC frequency (CFC/10^6 MNC) increased after co-culture over BST-1^+ 3T3 as compared with controls (220 ± 36 versus 70 ± 18, mean ± S.D. from three experiments; p < 0.06). In the presence of 10 nM NBMPR the stimulatory effect of the BST-1^+ feeder was abrogated (50 ± 11 versus 58 ± 15). Thus, the effect of paracrinally produced cADPR on CFC appears to be mediated by a concentrative NT, sensitive to nanomolar concentrations of the NT inhibitors dipyridamole and NBMPR, suggesting involvement of cs and/or csg (9, 10).

A role of accessory mononuclear cells (e.g. via an increased production of cytokines) in the stimulatory effect of exogenously added cADPR on HP has been already ruled out, because it was observed on total MNC as well as on the CD34^+ subpopulation, which is markedly enriched in both the committed and early HP (5). To determine whether the uptake of cADPR by purified CD34^+ cells was also mediated by a dipyridamole and NBMPR-sensitive CNT, we isolated CD34^+ cells from CB MNC and incubated the purified subpopulation with exogenously added cADPR in the presence or absence of NBMPR. Steady presence of nanomolar cADPR, as measured following daily replacement of the medium with fresh medium supplemented with 20 nM cyclic nucleotide, proved as effective as low micromolar cADPR in stimulating colony output from CD34^+ cells (Fig. 3). The increase of colony number relative to control cultures was more evident at longer incubation times (1–2 weeks) than observed with 100 μM cADPR, which caused doubling of colony output after 24 h incubation (5). As the long term culture (≥1 week) of CD34^+ cells with the nucleoside transport inhibitor NBMPR proved cytotoxic, we decided to use a cADPR concentration in the order of tens of micromolar to observe a significant increase of colony output after 24 h incubation. Thus, cADPR priming of CD34^+ cells with the nucleoside transport inhibitor NBMPR proved cytotoxic, we decided to use a cADPR concentration in the order of tens of micromolar to observe a significant increase of colony output after 24 h incubation. Thus, cADPR priming of CD34^+ cells with 50 μM cyclic nucleotide for 24 h induced a 2-fold increase of CFC (Fig. 4A) and a 3-fold increase of the [Ca^2+]i, compared with controls (Fig. 4B). Addition of 10 nM NBMPR prevented both effects of cADPR, reducing colony output and [Ca^2+]i, to control values. NBMPR alone did not significantly modify colony growth or [Ca^2+]i, in CD34^+ cells (Fig. 4, A and B).

**DISCUSSION**

Native hemopoietic stroma expresses the ADP-ribosyl cyclase BST-1 (13). Autocrine production of cADPR in BST-1^+
cells should be significantly lower than in CD38\(^-\) cells due to the low specific activity of BST-1 compared with CD38. Indeed, the limited increase of the [cADPR], in BST-1\(^+\) stromal cell lines compared with the corresponding controls did not induce significant modifications of the [Ca\(^{2+}\)], (Table I) and consequently of the IFN-\(\gamma\) production (see "Results"). During LTC of HP over CD38\(^-\) stroma, growth inhibition by IFN-\(\gamma\) overcomes the stimulatory effect of cADPR, resulting in a marked reduction of colony output compared with control co-cultures, set over CD38\(^-\) stroma (Table II). Conversely, LTC over BST-1\(^+\) 3T3 significantly increases colony output, starting from the 2nd week of co-culture (Table II). The difference in colony output between the two co-culture settings (over BST-1\(^+\) versus control stroma) increases during the course of LTC. This observation is confirmed by similar results obtained with the other BST-1\(^+\) stromal cell line used in this study, COS cells (Table III). Thus, it appears that the extracellular cADPR concentration generated by BST-1\(^+\) feeders, although in a subnanomolar range, is sufficient to exert a stimulatory effect on CFC proliferation during long term culture. This conclusion prompted us to investigate the mechanism of CDFR uptake by CFC.

In several, non-hemopoietic mammalian cell lines extracellularly added cADPR is translocated through both equilibrative (ENT-2) and concentrative (CNT-2 and csg) nucleoside transporters (9, 10). Among the mammalian equilibrative nucleoside transporters known to date, ENT1 (sensitive to inhibition by nanomolar NBMPR and dipyridamole), only ENT-2 is involved in cADPR transport (9, 10). The large family of the concentrative nucleoside transporters, collectively representing nucleoside-Na\(^+\) symport systems, includes CNT1, CNT2, and CNT3, which are NBMPR-insensitive (29, 30), and two additional, not yet molecularly defined, transporters (cs and csg), which are inhibited by nanomolar NBMPR and micromolar dipyridamole (27, 28).

Dipyridamole and NBMPR inhibit the stimulatory effect of cADPR on CFC at nanomolar concentrations (Fig. 2); since ENT-1 has been shown to be incompetent for cADPR transport (9), this points to the NBMPR/dipyridamole-sensitive CNTs cs and csg as responsible for CDFR entry into CFC. A further consideration suggests a causal role for concentrative transporter(s) in the influx of cADPR into these hemopoietic progenitors; the extremely low cADPR concentration in the medium conditioned by both CD38\(^-\) and BST-1\(^+\) 3T3, which is in the sub-nanomolar range, is several orders of magnitude below the known \(K_m\) of the equilibrative transporter (ENT2) for cADPR, while they are still lower than, but closer to, the \(K_m\) of the CNTs for cADPR (9, 10). The intracellular cADPR concentration ([cADPR]) in CD38\(^-\) 3T3 co-cultured over a CD38\(^-\) feeder is 10\(^5\) times higher compared with the extracellular level, indicating that a concentrative transport occurs (11). The finding that NBMPR prevents the stimulatory effect of cADPR on colony output and on [Ca\(^{2+}\)], increase on CD34\(^+\) cells confirms the involvement of a concentrative NT in CDFR transport into primitive HP (Fig. 4).

The fact that a concentrative NT mediates influx of cADPR into CFC is in line with the presence, in the bone marrow microenvironment, of a low extracellular cyclase activity, as that displayed by BST-1. Two considerations suggest that the [cADPR] in the BM microenvironment may be even lower than that recorded in the medium conditioned by CD38\(^-\) feeder layers: (i) expression of BST-1, instead of CD38, on stromal mesenchymal cells (MSC) and (ii) dilution of BST-1\(^+\) MSC with other cyclase-negative cell types. The hormone-like concentration of cADPR provided by the BST-1\(^+\) feeder might cooperate with other intracellular calcium-releasing signal molecules (inositol 1,4,5-triphosphate and NAADP\(^+\)) (31–33) to induce growth-stimulatory Ca\(^{2+}\) signals in HP. In CD38\(^-\) 3T3 fibroblasts, cADPR was found to cooperate with inositol 1,4,5-triphosphate in the generation and propagation of Ca\(^{2+}\) signals following stimulation of P2Y purinergic receptors (34).

In conclusion, these results indicate that the continuous production of hormone-like concentrations of extracellular cADPR by a BST-1-positive stroma stimulates LTC-IC proliferation \(in vitro\). Doubling of colony output can also be obtained by the continuous, 2-week-long exposure to nanomolar, exogenously added cADPR (Fig. 3), or to micromolar pulse-added cADPR (Fig. 4A). This seems to be a recurring motif in cytokine function; the continuous supply of low concentrations of stimulatory cytokines reduces their effective concentration by 2–3 logs, compared with that required to produce similar effects by pulse addition (35). Similarly, the steady release of growth-inhibitory IFN-\(\gamma\) by a transduced feeder has been shown to substantially reduce the effective concentration of the cytokine as compared with its direct addition to the medium (36).

In this study, the concentrative transport of cADPR into HP is apparently responsible for the efficacy of subnanomolar extracellular cADPR concentrations in eliciting a biological effect on HP. Expression of BST-1, instead of CD38, on stromal cells is advantageous, as it reduces the autocrine effects of cADPR.
on stroma, preventing the production of IFN-γ while producing enough extracellular cADPR to elicit a stimulatory effect on HP growth.

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Concentrative Uptake of Cyclic ADP-ribose Generated by BST-1+ Stroma Stimulates Proliferation of Human Hematopoietic Progenitors

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