STRIKING DECREASE IN THE TOTAL PRECURSOR B-CELL COMPARTMENT DURING EARLY CHILDHOOD AS EVIDENCED BY FLOW CYTOMETRY AND GENE EXPRESSION CHANGES

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The number of circulating B-cells in peripheral blood plateaus between 2 and 24 months of age, and thereafter declines gradually. How this reflects the kinetics of the precursor B-cell pool in the bone marrow is of clinical interest, but has not been studied thoroughly in humans. The authors analyzed bone marrow (n = 37) from healthy children and adults (flow cytometry) searching for age-related changes in the total precursor B-cell compartment. In an age-matched cohort (n = 25)
they examined age-related global gene expression changes (Affymetrix) in unsorted bone marrow with special reference to the recombination activating gene 1, RAG1. Subsequently, they searched the entire gene set for transcripts correlating to the RAG1 profile to discover other known and possibly new precursor B-cell related transcripts. Both methods disclosed a marked, transient increase of total precursor B-cells at 6–20 months, followed by a rapid decrease confined to the first 2 years. The decline thereafter was considerably slower, but continued until adulthood. The relative composition of total precursor B-cells, however, did not change significantly with age. The authors identified 54 genes that were highly correlated to the RAG1 profile ($r \geq 0.9$, $p < 1 \times 10^{-8}$). Of these 54 genes, 15 were characteristically B-lineage associated like CD19, CD79, VPREB, EBF1, and PAX5; the remaining 39 previously not described as distinctively B-lineage related. The marked, transient increase in precursor B-cells and RAG1 transcriptional activity is not reflected by a similar peak in B-cells in peripheral blood, whereas the sustained plateau concurs in time.

**Keywords**  adults, B-cells, bone marrow, children, flow cytometry, gene expression

Normal bone marrow (BM) constitutes a rapidly regenerating, complex tissue of blood progenitor cells changing in relative composition with age. Macroscopically, fat replaces hematological active red marrow that retracts to the axial skeleton in adults [1]. In healthy individuals, the capacity to produce all blood cell components throughout life is preserved, though a functional decline in the immune system is seen with advancing age [2]. The absolute number of circulating peripheral B cells has been shown to plateau between 2 and 24 months of age, then gradually decline [3].

Differentiation of precursor B-cells in the BM is dependent on the assembly of immunoglobulin (Ig) gene components, a process regulated by the lymphocyte-specific recombination activating genes RAG1 [4] and RAG2 [5]. RAG genes are also involved in the assembly of T-cell receptor genes in developing thymic T cells [6], but are not expressed in peripheral T-lymphocytes. Hence, in BM, RAG1 and RAG2 are uniquely linked to developing B cells.

The aim of the present study was to search for age-related changes in the total precursor B-cell compartment by two different methods, flow cytometry and gene expression profiling, and to examine the compositional pattern of precursor B-cell subpopulations with age. Furthermore, by using the age-related RAG1 expression profile as a reference transcript for precursor B cells and correlation analysis to RAG1, we wanted to discover other known and potentially new precursor B-cell-related transcripts.

**METHODS**

**Bone Marrow from Healthy Individuals**

We obtained BM samples from 63 healthy individuals aged 1 month to 41 years, and analyzed 37 of those with multiparameter flow cytometry (32 children, 5 adults) and 25 with gene expression profiling (20 children, 5 adults). The children were eligible for minor surgery, and the adults were
TABLE 1 The set of monoclonal antibodies used for the selected quadruple immunostainings to separate precursor B-cell subsets in normal bone marrow

| Combination | CD  | Clone | Fluorochromes | Manufacturer |
|-------------|-----|-------|---------------|--------------|
| 1           | CD10| HI10A | FITC          | BD Biosciences |
| CD20        |     | L27   | PE            | BD Biosciences |
| CD19        |     | J4.119| PECy5         | Immunotec     |
| CD45        |     | 2DI   | APC           | BD Biosciences |
| 2           | CD34| 8G12  | FITC          | BD Biosciences |
| CD22        |     | BL-CAM| PE            | BD Biosciences |
| CD19        |     | J4.119| PECy5         | Immunotec     |
| CD45        |     | 2DI   | APC           | BD Biosciences |

voluntary health care workers. Written informed consent was obtained using protocols approved by the Regional Medical Research Ethics Committee of Eastern Norway (REK Øst). The study was performed according to the Norwegian Health Regulations and Law of Biobanking.

Composite BM samples (2.5 mL) were used for the gene expression analysis by aspiration and immediately transferred to PAXgene tubes (PreAnalytiX, Hombrechtikon, Switzerland) for mRNA stabilization [7]. Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), and RNeasy (Qiagen, Hilden, Germany). RNA was quantified by absorbance spectrophotometry at 260 nm by NanoDrop ND-1000 (Saveen Werner, Malmö, Sweden). RNA quality was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

**Multiparameter Flow Cytometric Immunophenotyping**

BM samples were studied using a panel of monoclonal antibodies directly conjugated with fluorochromes as summarized in Table 1. Briefly, washed BM (50 µL) was stained (20 min) with saturating concentrations of monoclonal antibodies (MAbs), erythrocytes were lysed (FACS Lysing Solution, BD, 15 min), and the remaining cells were washed (phosphate-buffered saline) and fixed in 500 µL 1% w/v paraformaldehyde according to the manufacturer’s instruction. Flow cytometric analysis was performed using a Cyan ADP (Dako, CA, USA) equipped with a 488-nm and a 635-nm laser. For each antibody combination, 100,000 cellular events were acquired. Summit software (Dako, Santa Barbara, CA, USA) was used for data analysis.

The different B-cell populations were estimated as shown in Figure 1. Pro-B cells were CD34+CD22+CD19−, pre-B I cells were CD19+CD10+CD20−CD45−weak, pre-B II large cells were CD19+CD10+CD20−CD45−dim, pre-B II small cells were CD19+CD10+CD20−CD45−dim, immature B-cells were CD19+CD10+CD20−bright CD45+, and mature B-cells were CD19+CD10−CD20+CD45+. 
FIGURE 1 Age-related (A) size of the total precursor B-cell compartment and (B) RAG1 transcriptional profile in normal BM. (A) Age-related size of the total precursor B-cell compartment (defined as the sum of CD19⁺CD10⁺ and CD19⁻CD22⁺CD34⁺ cells) in percentage of all BM nucleated cells from another cohort of 37 healthy children, adolescents, and adults. The peak in precursor B-cells was observed at 6 months. The overall age-related pattern of RAG1 expression was comparable with the profile of the total precursor B-cell compartment. (B) Age-related RAG1 expression from DNA microarray analysis of composite BM from 25 healthy children, adolescents, and adults. Peak expression values for RAG1 was seen at about 3–20 months of age.

Gene Expression Profiling

cDNA and cRNA synthesis and labeling were performed according to the recommended Affymetrix protocols (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). The solutions were hybridized, washed, and stained using Affymetrix HG-U133 Plus 2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA, USA) covering 54,675 probe ids and scanned using Affymetrix Gene Chip Scanner 3000. Signal values were scaled by global methods to a target value of 100. The arrays had an average
GAPDH 3′/5′ ratio of 1.11 (SD ± 0.1, range 0.94–1.36) and an average percent present call of 40.5% (SD ± 2.5, range 32.9–43.8%). The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE11504.

Statistical Analysis

Robust Multichip Average (RMA) was used to convert the Affymetrix annotated raw signal strength values to normalized expression values [8,9]. RMA consists of a three-step approach, which uses a background correction on the Perfect Match (PM) data, quantile normalization and summarizes the probe set information by using Tukey’s median polish algorithm. The 10,000 transcripts most correlated to RAG1 as a function of age were found using the Pearson correlation metric in the gene filter function of the Bioconductor project and the R program free software (http://www.bioconductor.org/). These 10,000 coefficients were recalculated using the Tukey’s biweight similarity metric [10], which is more robust to outlying signal values found in noisy microarray data. The permutation \( p \) values associated with these profiles were calculated by using the 10,000 correlated profiles, thereby permuting the samples 10,000 times, followed by calculation of the Pearson correlation coefficients, and counting the number of profiles at a correlation level of interest. Therefore, the reported permutation \( p \) values represent the probability of seeing a randomly generated gene with a correlation to RAG1 of the chosen correlation level or higher. The probability of seeing more than one randomly generated gene correlated to RAG1 would be less than reported. Gene ontology analysis was performed with the David v2008 ontology program (http://david.abcc.ncifcrf.gov/summary.jsp) and the Ingenuity Pathway Analysis 3.0 software (IPA) (Ingenuity Systems, www.ingenuity.com).

RESULTS

Changes in the Precursor B-cell Compartment as a Function of Age and Its Relation to the RAG1 Transcriptional Profile

The size of the precursor B-cell compartment, defined as the sum of CD19\(^+\)CD10\(^+\) and CD19\(^-\)CD22\(^+\)CD34\(^+\) cells in percentage of the total BM cells, increased during the first months of life to maximum 18.6% at 6 months (Figure 1A). From then on, a rapid decline followed to an average value of 3.6% at about 10 years, and then further decreasing to average 0.8% in adults. For both cohorts the initial decline represented approximately 80% of the total reduction. Maximal expression of RAG1 was found at 20
FIGURE 2 Flow cytometric analysis of BM showing normal B-cell differentiation. Estimation of pro-B cells (C) was done within the lymphogate (A) on CD34+ (B) and CD22+CD19− cells (C). The composition of the CD19+ B-cell compartment was analyzed within the lymphogate. CD19+ B-cell subsets (D) were gated on CD45 vs. CD10 (E) and CD10 vs. CD20 markers (F). They appear as pre-B I (red), pre-B II small (green), pre-B II large (pink), immature B (yellow), and mature B (blue). Pre-B II small cells are partly hidden behind the pre-B II large cells in the CD45 vs. CD10 diagram.

months of age, then decreased rapidly till about 39 months, followed by a much slower decline thereafter (Figure 1B).

We further analyzed the composition of the precursor B-cell compartment (Figures 2 and 3) to explore if it differed with age, especially if shifts in highly RAG1 expressing subsets (pre-B I and pre-B II small) did occur. We found no significant age-related shift by linear regression analysis in any of the 5 precursor B-cell subsets—pro-B, pre-B I, pre-B II large, pre-B II small, or immature B-cells—that could explain the decrease in RAG1 expression. Based on regression analysis the correlation coefficients were $r = .147 (p = .378)$ for pro-B, $r = .84 (p = .617)$ for pre-B I, $r = .07 (p = .968)$ for pre-B II large, $r = .102 (p = .544)$ for pre-B II small, and $r = .90 (p = .592)$ for immature B-cells. Subsets within the total precursor B-cell compartment given as mean and range values: pro-B 4.0% (0–9.61%), pre-B I 6.8% (2.8–13.3%), pre-B II large 35.1% (16.2–53.5%), pre-B II small 23.6% (10.8–39.4%), and immature B-cells 32.8% (13.6–64.0%).

Although representing two different cohorts, the consistency between the size of the total precursor B-cell compartment and the RAG1 expression
profile implies that the decrease in \textit{RAG1} expression might serve as an indicator for a decrease in the precursor B-cell compartment.

\textbf{Results from Global Gene Expression Profiling}

To search for transcripts whose expression profiles were highly correlated to the age-dependent \textit{RAG1} profile, we chose to use the whole Affymetrix data set of 54,675 probe-ids. Submitted to annotation analysis in IPA, we found that 45,845 of the transcripts were eligible for functional mapping and 11,249 qualified for networks. Of them, 490 genes mapped to networks involved in hematological and/or immunological processes and 3660 represented other networks.

\textbf{Transcripts Showing an Age-Related Expression Profile Highly Correlated to \textit{RAG1}}

By applying Tukey’s biweight correlation analysis we identified 78 probe-ids representing 54 annotated genes that significantly correlated to the age-related \textit{RAG1} profile with a correlation coefficient \( r \geq .9 \) and \( p \) value \(< 1 \times 10^{-8} \). The 54 genes are listed in Table 2 and displayed as a heat map in Figure 4 using gene expression values scaled to the median expression.
| Entrez ID | Gene title       | Affy ID       | Gene name                                                                 |
|-----------|------------------|---------------|---------------------------------------------------------------------------|
| 29760     | BLNK             | 207655_at     | B-cell linker                                                             |
| 930       | CD19             | 206598_at     | CD19 antigen                                                              |
| 971       | CD72             | 215925_at     | CD72 antigen                                                              |
| 973       | CD79A            | 205049_at     | CD79a antigen, immunoglobulin-associated alpha                            |
| 974       | CD79B            | 205297_at     | CD79b antigen, immunoglobulin-associated beta                             |
| 4064      | CD180            | 206206_at     | CD180 antigen                                                             |
| 1791      | DNTT             | 210487_at     | Deoxynucleotidyltransferase, terminal                                      |
| 1879      | EBF1             | 232204_at     | Early B-cell factor 1                                                     |
| 3535      | IGL8             | 230128_at     | Immunoglobulin lambda locus                                               |
| 3543      | IGLL1            | 206660_at     | Immunoglobulin lambda-like polypeptide 1 (λ5)                            |
| 5079      | PAX5             | 1566428_at    | Paired box gene 5 (B-cell lineage specific activator protein)            |
| 5450      | POU2AF1          | 205267_at     | POU domain, class 2, associating factor 1                                 |
| 5897      | RAG2             | 215117_at     | Recombination activating gene 2                                           |
| 7441      | VPREB1           | 221349_at     | Pre-B lymphocyte gene 1                                                   |
| 29802     | VPREB3           | 220068_at     | Pre-B lymphocyte gene 3                                                   |

Genes with known B-cell association

| Entrez ID | Gene title       | Affy ID       | Gene name                                                                 |
|-----------|------------------|---------------|---------------------------------------------------------------------------|
| 9590      | AKAP12           | 210517_at     | A kinase (PRKA) anchor protein (gravin) 12                                 |
| 60488     | BACH2            | 221234_at     | BTB and CNC homology 1, basic leucine zipper transcription factor 2        |
| 605       | BCL7A            | 203787_at     | B-cell CLL/lymphoma 7A                                                    |
| 199786    | BCNP1            | 230983_at     | B-cell novel protein 1                                                     |
| 817       | CAMK2D           | 224994_at     | Calcium/calmodulin-dependent protein kinase (CaM kinase) II delta          |
| 928       | CD9              | 236085_at     | CD9 antigen                                                               |
| 55179     | FAIM             | 220643_at     | Fas apoptotic inhibitory molecule                                         |
| 84824     | FCRLA            | 235372_at     | Fc receptor-like and mucin-like 1                                         |
| 2841      | GPR18            | 210279_at     | G protein-coupled receptor 1                                              |
| 3108      | HLA-DMA          | 217478_at     | Major histocompatibility complex, class II, DM alpha                      |
| 3122      | HLA-DRA          | 210982_at     | Major histocompatibility complex, class II, DR alpha                      |
| 8739      | HRK              | 206864_at     | Harakiri (BCL2-interacting protein)                                       |
| 3662      | IRF4             | 204562_at     | Interferon regulatory factor 4 (Lymphocyte specific IRF)                   |
| 6659      | SOX4             | 201417_at     | SRY (sex determining region Y)-box 4                                      |
| 6925      | TCF4             | 228837_at     | Transcription factor 4 (Immunoglobulin transcription factor 2)            |
| 8115      | TCL1A            | 209995_at     | T-cell leukemia/lymphoma 1A                                                |

Genes with a supposedly widespread expression

| Entrez ID | Gene title       | Affy ID       | Gene name                                                                 |
|-----------|------------------|---------------|---------------------------------------------------------------------------|
| 323       | APBB2            | 212970_at     | Amyloid beta (a4) precursor protein-binding, family b, member 2 (fe65-like) |
| 84159     | ARID5B           | 212614_at     | AT rich interactive domain 5B (MRF1-like)                                  |
| 79161     | C7orf23          | 204215_at     | Chromosome 7 open reading frame 23                                        |
| 90488     | C12orf23         | 224759_at     | Chromosome 12 open reading frame 23                                        |

(Continued on next page)
and ordered by increasing age. (See Additional File 1 for all transcripts that correlated to RAG1 with \( r \geq 0.7 \) and \( p < 3 \times 10^{-4} \).)

**Functional Annotations of Transcripts Showing Co-variation with RAG1 Expression**

For the 54 RAG1 correlated genes, the 6 central biological functions \((p < 0.05)\) as defined by pathway analysis with IPA were (1) cellular development, (2) hematological system development and function, (3) immune and lymphatic system development and function, (4) tissue morphology, (5) cell growth and proliferation, and (6) immune response. The canonical pathways in IPA depicted B-cell receptor signalling as the most significant pathway \((p < 0.0001)\), despite the fact that only 5 of the 148 pathway genes were in the input significant gene list. This implies a high statistical correlation between B-cell receptor signalling and the 5 transcripts CD19, CD79A, CD79B, BLNK, CAMK2D.
FIGURE 4 Heat map of transcripts highly correlated to the age-related RAG1 profile. The heat map illustrates the overall age-related decline in gene expression for RAG1 and 54 highly correlated transcripts in composite BM. Each horizontal lane represents a transcript and each vertical lane a study subject identified by age in months. Probe set signal values were normalized with the RMA algorithm as explained in Materials and Methods. The relative expression for each individual at a given probe set is represented by a color (red signifying high expression and blue low expression).
As shown in Table 2, the 54 transcripts that highly correlated to the \textit{RAG1} profile \((r \geq .9)\) comprised (1) genes that are restricted to or preferentially expressed in B-lineage cells \((n = 15)\), (2) genes with known B-lineage association \((n = 16)\), and (3) genes with a supposed broader tissue expression \((n = 23)\).

1) The genes well documented as either B-lineage restricted or with a preferential expression in B-lineage cells, coded for proteins localized to the plasma membrane, cytosol or nucleus (Figure 5). The transcripts represented B- and precursor B-cell surface protein markers \((CD19, CD72, CD79A, CD79B, CD180, IGLL1, IGL@, VPREB1, VPREB3)\), a signal transduction molecule \((BLNK)\), transcription factors \((EBF1, PAX5, POU2AF)\) and proteins directly involved in V(D)J recombination \((DNTT, RAG2)\) [12]. Accordingly, all these 15 transcripts are considered key players in normal precursor B-cell development and function.
2) Among the genes with recognized B-lineage association a few candidates can be mentioned: \textit{BCNP1} is a newly identified protein with unknown function highly expressed in lymph nodes and spleen, less in thymus. Its expression is retained and often upregulated in B-cell malignancies [13]. \textit{HRK} is recently described as a pro-apoptotic member of the Bcl-2 family. \textit{HRK}'s role in the regulation of normal hematopoiesis is unknown, but it is specifically and rapidly induced in hematopoietic progenitors after growth factor deprivation or chemotherapeutic treatment [14].

3) The last group of transcripts consists of supposedly widely expressed molecules involved in general cellular functions. \textit{PCDH9} belongs to the cadherin superfamily genes primarily expressed in the central nervous system. Interestingly, the protocadherin subfamily, to which \textit{PCDH9} belongs, displays unusual genomic structures reminiscent of Ig and T-cell receptor genes [15]. Notably, the expression of all these transcripts followed closely the \textit{RAG1} profile in our normal BM material.

**DISCUSSION**

Presently, the knowledge regarding age-related precursor B-cell production in human BM is unclear. We aimed to show by flow cytometry how the precursor B-cell compartment changes with age in a cohort of healthy infants, children, and middle-aged adults. In addition, we examined the age-related profile of V(D)\textit{J} recombination activating gene, \textit{RAG1} in composite BM from a separate comparable cohort. Furthermore, we searched for genes demonstrating the same age-dependent profile as \textit{RAG1} in the global gene expression data.

The size of the total precursor B-cell compartment decreased with age, reflecting a parallel reduction in all analyzed subsets when related to the number of total BM cells. Consequently, no age-related changes within the precursor B-cell compartment could be detected to explain the variation in \textit{RAG1} levels consistent with a previously published study [16]. Accordingly, \textit{RAG1} expression in total BM could be used as a marker of the total precursor B-cell compartment. We found a significant correlation between the \textit{RAG1} profile and 15 additional precursor B-cell characteristic transcripts, lending support to our hypothesis to use the global gene expression data set to estimate relative precursor B-cell abundance.

The decline of the total precursor B-cell pool in the bone marrow correlates well with the decline in the absolute number of B-cells in peripheral blood [3]. The two compartments seem to follow the same kinetics, although the changes in peripheral blood lags slightly behind the bone marrow results, and the initial precursor B-cell peak could not be found. The expression patterns of \textit{RAG1} (and \textit{RAG2}) have been shown by others [11] to peak in pre-B-I and pre-B-II small cells, the population stages in which complete Ig
Early Decline in the Precursor B-cell Pool

Heavy and light-chain gene rearrangements are formed, respectively, and further to decrease significantly in immature B cells [17].

With this approach we not only confirmed known precursor B-cell transcripts, but were able to identify a limited number of genes that were highly correlated to the RAG1 expression profile \((r \geq .9, p < 1 \times 10^{-8})\) with hitherto no established B-lineage annotation. To identify those that belong to the precursor B-cell subsets, gene expression profiling on sorted subpopulations of precursor B-cells should be carried out.

We next raised the question as to what extent our set of genes that were strongly correlated to RAG1, appeared in the list of B-lineage transcripts previously defined by others. First, we correlated our extended set of genes (Additional File 1, age-related profile like RAG1, \(r > .70\),) to the 243 genes identified by van Zelm et al.\(^{11}\) being transcripts, which correlated with the initiation of Ig gene rearrangements in precursor B-cells. We found 24 transcripts (median and mean \(r = .84\)): 18 DNA-binding transcription factors (AEBP1, ARID5B, BACH2, CXXC5, ETS2, FOXO1A, IRI\(^4\), MEF2C, PAX5, POU4F1, SOX4, SMAD1, SPIB, UHRF2, ZBTB33, ZCCHC7, ZHX2, ZNF608), 1 gene involved in DNA methylation (DNMT3B), 1 chromatin and histone remodeling gene (HDAC9), 2 genes involved in recombination (TdT or DNTT and RAG2), and 2 genes involved in DNA repair (CSNKIE and NEIL1).

Second, we compared our results to those of Palmer et al.[18], who correlated gene expression of isolated subpopulations to relative cell abundance in whole blood and defined for B cells a signature of 427 genes. Compared to their list, we found an overlap of 22 genes using our list of 54 transcripts \((r > .90)\). Furthermore, we looked to see if the top 5 T-cell correlated transcripts from the list of Palmer et al. (CD3D, CD3G, LEF1, MAL, TCF7) changed with age in our material. We found no significant correlation with age for these T-cell signature genes (results not shown), and no overlap between our genes showing a RAG1 like profile and their T-cell signature transcripts, suggesting that the RAG1 correlated mRNAs are not T-cell associated.

Gene expression studies on composite BM may be confounded by variations in the relative proportions of cells, but do represent a true picture of the total marrow activity after minimal sample manipulation, thus reducing in vitro transcriptional changes. Elucidating the complexity and molecular interactions in composite BM seems to be an inevitable step in understanding both normal changes occurring with age as well as malignant transformation \([19]\).

In conclusion, we found that the decline in the precursor B-cell compartment not only is initiated in early childhood, but primarily takes place during the first two years of life. It can only be speculated whether the early transient peak in precursor B cells is caused by feedback from the periphery or reflects a BM intrinsic mechanism. Finally, we provide a platform for information regarding transcriptional changes in healthy human BM from infancy to young adult age. The complete age-related BM gene expression...
material is available online at Gene Expression Omnibus (GEO), GEO Series accession number GSE11504, (http://www.ncbi.nlm.nih.gov/geo/).

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Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Additional file 1. Transcripts correlated to RAG1 ($r > .70$, $p < 3 \times 10^{-4}$). The excel sheet shows the 675 transcripts that were correlated to RAG1 with decreasing correlation coefficient until $r > .70$ using the Tukey’s biweight correlation analysis. Shown are Affymetrix probe id numbers, gene symbols and RefSeq Transcript ids.

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