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Macrophages and their subpopulations following allogeneic bone marrow transplantation for chronic myeloid leukaemia

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Abstract A morphometric and immunohistochemical study was performed on 354 bone marrow trephine biopsies derived from 126 patients with chronic myeloid leukaemia (CML) before and after allogeneic bone marrow transplantation (BMT). The purpose of this investigation was to evaluate the macrophage population, including several subsets and their dynamics in the post-transplant period. In addition to the total $CD68^+$ resident (mature) macrophages the so-called activated fraction identified by its capacity to express α-D-galactosyl residues, the pseudo-Gaucher cells (PGCs) and the iron-laden histiocytic reticular cells were also considered. Following immuno- and lectin-histochemical staining morphometric analysis was carried out on sequential postgraft bone marrow specimens at standardized intervals. Compared to the normal bone marrow and calculated per haematopoiesis (cellularity) an overall decrease of about 40–50% in the quantity of $CD68^+$ macrophages and the $BSA\text{-}I^+$ subpopulation was detectable in the early post-transplant period (9–45 days after BMT). Noteworthy was the temporal recurrence of PGCs in the engrafted bone marrow, which was not associated with a clonally transformed cell population or leukaemic relapse. Reappearance of postgraft PGCs was most prominent in the first 2 months after BMT. This conspicuous feature was presumed to be functionally associated with a pronounced degradation of cell debris following pretransplant myelo-ablative therapy (scavenger macrophages). Evidence for an activation of the $BSA\text{-}I^+$ macrophage subset was derived from the identical carbohydrate-binding capacity shown by the PGCs. In the regenerating haematopoiesis shortly after BMT a significant correlation between the number of $BSA\text{-}I^+$ macrophages and erythroid precursor cells was determinable. This result implicates a close functional relationship between postgraft reconstitution of erythropoietic islets and centrally localized activated macrophages. In conclusion, findings emerging from this study included the reappearance of PCGs in the engrafted bone marrow independently of a leukaemic relapse and the significant association of the activated $BSA\text{-}I^+$ macrophage subset with the recovery of erythropoiesis.

Key words Macrophages · Pseudo-Gaucher cells · Chronic myeloid leukaemia · Bone marrow transplantation · Bone marrow biopsies

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Introduction

Although in recent years considerable progress has been made concerning the biology of allogeneic stem cell and bone marrow transplantation (BMT) in Philadelphia chromosome-positive chronic myeloid leukaemia (CML), so far little information is available on morphological changes associated with pretransplant myelo-ablative therapy or reconstitution of normal haematopoiesis [5, 8, 9, 14]. With the exception of myelofibrosis [30] hardly anything is known about the other constituents of the bone marrow stroma responsible for the complex functional network composing the microenvironment, which exerts a significant impact on haematopoietic recovery and differentiation [23]. In this elaborate concert of various cell-to-cell and stromal matrix interactions, the resident macrophages deserve special attention. In CML the latter have long been recognized as a potentially important cell population, particularly from the aspect of the expansion of the leukaemic cell clone, which may be mediated by malignant stromal macrophages [6]. In addition to their clonal transformation [13, 33], resident macrophages of the CML bone marrow are further char-

acterized by a striking diversity of cytological appearance revealing the peculiar phenomenon of acquired lipidosis [10, 15]. Among other cell types, this feature generates the pseudo-Gaucher cells (PGCs) and the so-called sea-blue or blue-pigmented histiocytes [2, 7, 18, 20, 31, 34]. Moreover, there is also a conspicuous functional heterogeneity of the mature macrophages showing a subset with α-D-galactosyl residue-binding carbohydrate compounds on their surface [4, 22, 34, 39]. Following in vitro studies, this reactivity is compatible with an activation-associated antigen [1, 16, 19]. In keeping with the hypothesis that occurrence of PCGs is linked with a pronounced turnover of granulocytes and erythrocytes [10, 15, 18], it is speculated that this specific cell fraction might also express corresponding binding with the lectin from Bandeiraea (Griffonia) simplicifolia isotype I-B_4 specific for this sugar moiety [4, 22, 39]. The purpose of the present immunohistochemical and morphometric study was to analyse the (total) $CD68^+$ resident macrophage population and its activated fraction including PGCs before and after allogeneic BMT in CML. For this reason, 354 repeated pre- and postgraft trephine biopsies derived from 126 patients were evaluated at standardized endpoints to elucidate (1) quantitative changes of the mononuclear-macrophage compartment, (2) possible retrieval of PGCs, and (3) putative associations with haematological parameters indicative of engraftment.

Patients and methods

Patients

The series followed in this study consisted of 126 patients (74 male, 52 female; median age at BMT 35 years), who had presented in the first phase of chronic (stable) CML and were retrospectively analysed. In the course of allogeneic BMT patients received marrow grafts from HLA-identical family members or other donors at a single referral transplantation centre following standard procedures. Details of the history, clinical findings and treatment characteristics of each patient have been reported in detail elsewhere [5]. Following BMT a successful engraftment according to standard criteria [5, 8, 9, 14] was established in 95 patients and delayed haematopoietic reconstitution in 31 patients.

Bone marrow biopsies

Representative bone marrow trephine biopsies (mean size 17.5×1.8 mm) were performed from the posterior iliac crest at standardized intervals during the course of the transplant procedure (Table 1). Fixation of samples was carried out in a phosphate-buffered aldehyde solution at a low concentration (2–3%) for 12–48 h. Further processing included decalcification for 3–4 days in 10% buffered ethylene-diamine tetra-acetic acid (EDTA), pH 7.2, paraffin embedding, and several routine staining techniques, including Giemsa, PAS (periodic acid–Schiff reagent), naphthol-AS-D-chloroacetate esterase, Perls' reaction for iron, and a silver impregnation method (Gomori's technique). For specific staining of the mature $CD68^+$ macrophage population the monoclonal antibody PG-M_1 [11, 31] was applied. For the labelling of the activated subset, their ability to express α-D-galactosyl residues which caused a specific binding to a lectin from Bandeiraea (Griffonia) simplicifolia (BSA-I) was used [4, 22, 34, 39]. The specific macrophages with excessive lipid storage, the PGCs, were further distinguished not only by an onion-skin like pattern following PAS reaction, but also by their positive birefringence in Giemsa-stained specimens [2, 7, 10, 15, 18]. To evaluate the activity of the BSA-I^+ macrophage fraction in relation to the posttransplant reconstitution of erythropoiesis a monoclonal antibody against glycophorin C (Ret 40f) was applied for clear-cut identification of nucleated erythroid precursor cells [12]. However, for a proper correlation, determination of this parameter was limited to the 25 patients who had a biopsy performed shortly after BMT (endpoint II in Table 1). Monoclonal antibodies and other reagents were purchased from Dako-Diagnostica (Hamburg, Germany). Details of staining procedures (APAAP method) were reported in previous communications [4, 11, 12, 31].

Morphometry

Following immunostaining, morphometric analysis was performed by two manual optic planimeters (MOP-A-MO1-Kontron and VIDAS-Zeiss-Kontron) with a standard program set (Kontron software) on large trephine biopsies with an artefact-free mean marrow area of 14.8±4.2 mm^2. Frequencies of $CD68^+$ and BSA-I^+ macrophages, and also erythroid precursors (in a selected number of posttransplant specimens), were calculated per square millimetre of bone marrow by examination of the total biopsy specimen and the areas occupied by haematopoiesis (cellularity). Reference to cellularity was necessary first to focus on the areas directly involved in haematopoietic regeneration and secondly to avoid the erroneous impression of a significant reduction in macrophage numbers following a pronounced expansion of adipose tissue or interstitial oedema. This feature was prominent particularly after marrow-ablative therapy and during the posttransplant period. Because PGCs and iron-laden macrophages were present only in a certain fraction of patients and represented a subset of the total $CD68^+$ macrophage population, their incidence was determined not by morphometry, but by semiquantitative grading. Scoring was as follows: 0 absence, + single to few scattered, ++ low to moderate number, +++ conspicuous increase with clustering. In view of the significantly different intervals between the 354 sequential biopsies in our series of 126 patients, calculations of changes regarding these variables had to be carried out by computing standardized endpoints, particularly after BMT (Table 1). For this reason, morphometric analysis was focused on these defined endpoints characterizing short intervals during the posttransplant period, and thus did not take account of the total of more than 500 evaluable bone marrow specimens. Statistical evaluation included a paired-samples T-test to assess changing patterns of pre- and postgraft macrophage populations (Table 1).

Results

Mature $CD68^+$ macrophages revealed a random distribution in the CML bone marrow and were of a roughly stellate shape (Fig. 1a). Occasionally they displayed an obvious phagocytic activity with engulfment of haematopoietic cells, mostly erythrocytes or normoblasts and a variety of other cell debris (Fig. 1b). On gross evaluation the incidence of activated BSA-I^+ macrophages seemed to be lower than in the total $CD68^+$ population. PGCs were easily recognizable in Giemsa-stained specimens by the pale, silver foil- or greaseproof-like appearance of their cytoplasm and the diagnostic positive birefringence following polarization. Moreover, the PAS reaction revealed a specific crushed tissue paper- or onion skin-like pattern (Fig. 1c), and there was distinctive positive staining with BSA-I (Fig. 1d). In addition to these characteristic features, a prevalent localization at the paratrabecu-

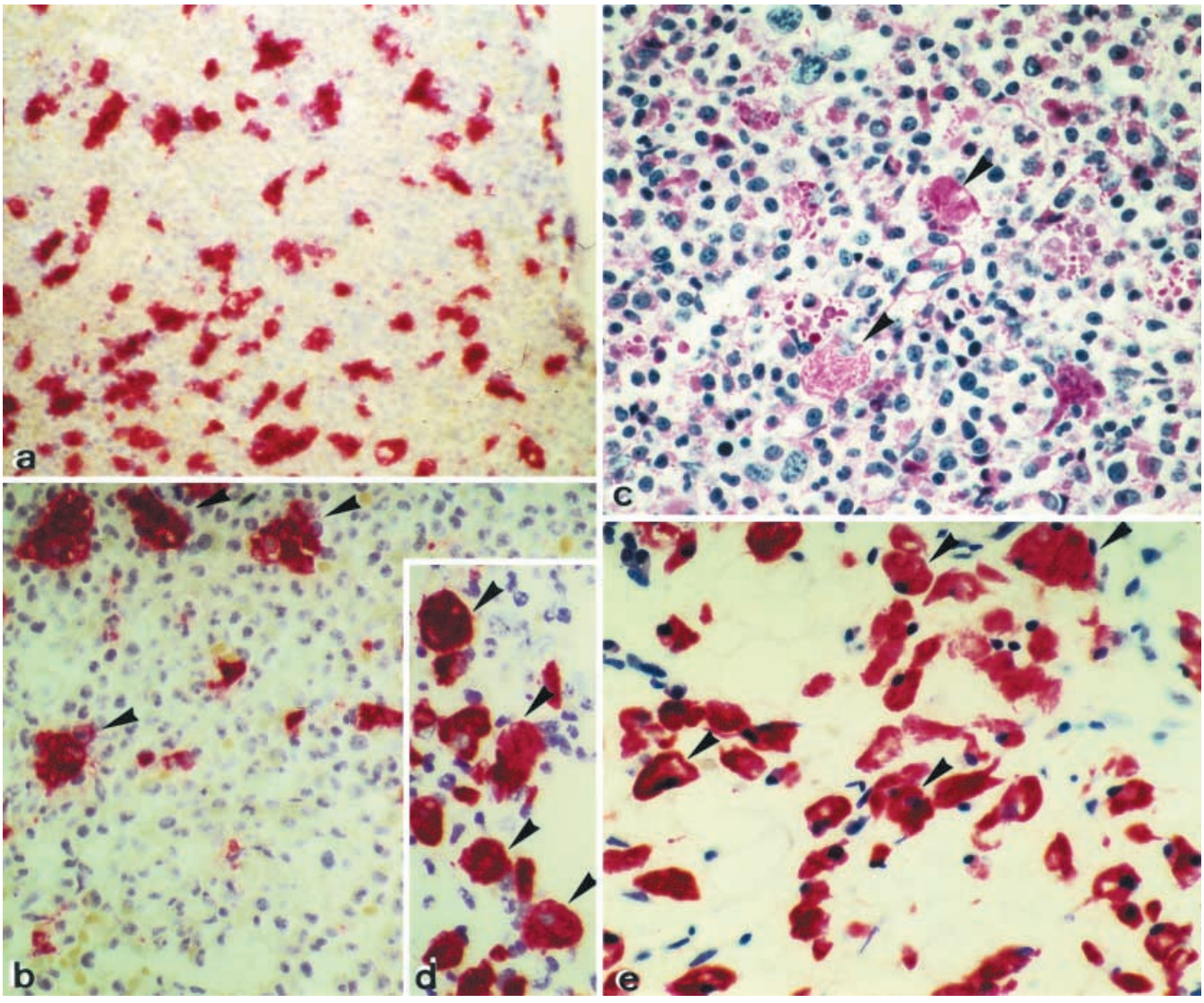


Fig. 1a–e Macrophages in the CML bone marrow **a–d** shortly before and **e** after BMT. **a** CD68$^+$ macrophages reveal a randomly dispersed distribution throughout the marrow space. **b** Ingestion of nuclear debris in large irregularly shaped macrophages (*arrowheads*). **c** Pseudo-Gaucher cells (PGCs) with distinctive onion skin- or crushed tissue-paper-like cytoplasmic pattern (*arrowheads*). **d** Corresponding cells reveal BSA-I reactivity (*arrowheads*) before BMT. **e** Loose clusters of BSA-I$^+$ (activated) macrophages including PGCs (*arrowheads*) shortly after BMT. **a** CD68, ×170; **b** CD68, ×370; **c** PAS, ×370; **d**, **e** BSA-I, ×370

lar (endosteal) areas of the bone marrow space was seen. In pretransplant biopsies considerable variation in the incidence of macrophages was encountered (Table 1). Following myelo-ablative regimens and BMT quantification of the engrafted CD68$^+$ and BSA-I$^+$ macrophage population per total bone marrow area at the standardized endpoint intervals disclosed no significant differences (paired samples T-test: $P>0.05$) from the pretransplant amount (Table 1). In contrast to these rather inconspicuous changes between the recipient and donor patients, compared to normal bone marrow features a significant decrease of about 40–50% in the macrophage population per area of haematopoiesis (cellularity) was determinable in the postgraft specimens 9–45 days after BMT (Table 1). Moreover, in a number of patients the BSA-I$^+$ activated macrophages frequently consisting of PGCs showed a pronounced increase in the posttransplant specimens (Fig. 1e). This enhancement not only involved an incidence in the donor bone marrow that was comparable to that in the recipient, but often corresponded to grade +++ on semiquantitative evaluation (Fig. 2a, b). A prominent arrangement in small loose (Fig. 2b), or occasionally in dense and extensive, clusters was detectable, in particular shortly after BMT (Fig. 2c, d). Reappearance of PGCs in the posttransplant bone marrow was an early event, starting as soon as on day 10, and became most prominent in the first 2 months after BMT. Moreover, the temporary retrieval of this particular cell population proved to be dependent mainly on their former presence in the CML bone marrow of the recipient. However, in this context PGCs were not associated with a clonal transformation or leukaemic relapse, as shown by the

Table 1 Endpoints of bone marrow examinations at standardized intervals during transplantation procedures for CML patients. Morphometric analysis of the macrophage population (mean±SD) was carried out per square millimetre of total bone marrow and haematopoiesis (*in brackets*) and included certain subsets and their relative frequency. Any one patient might have up to five sequential biopsies. Data on normal bone marrow were partially derived from previous studies [4, 34]

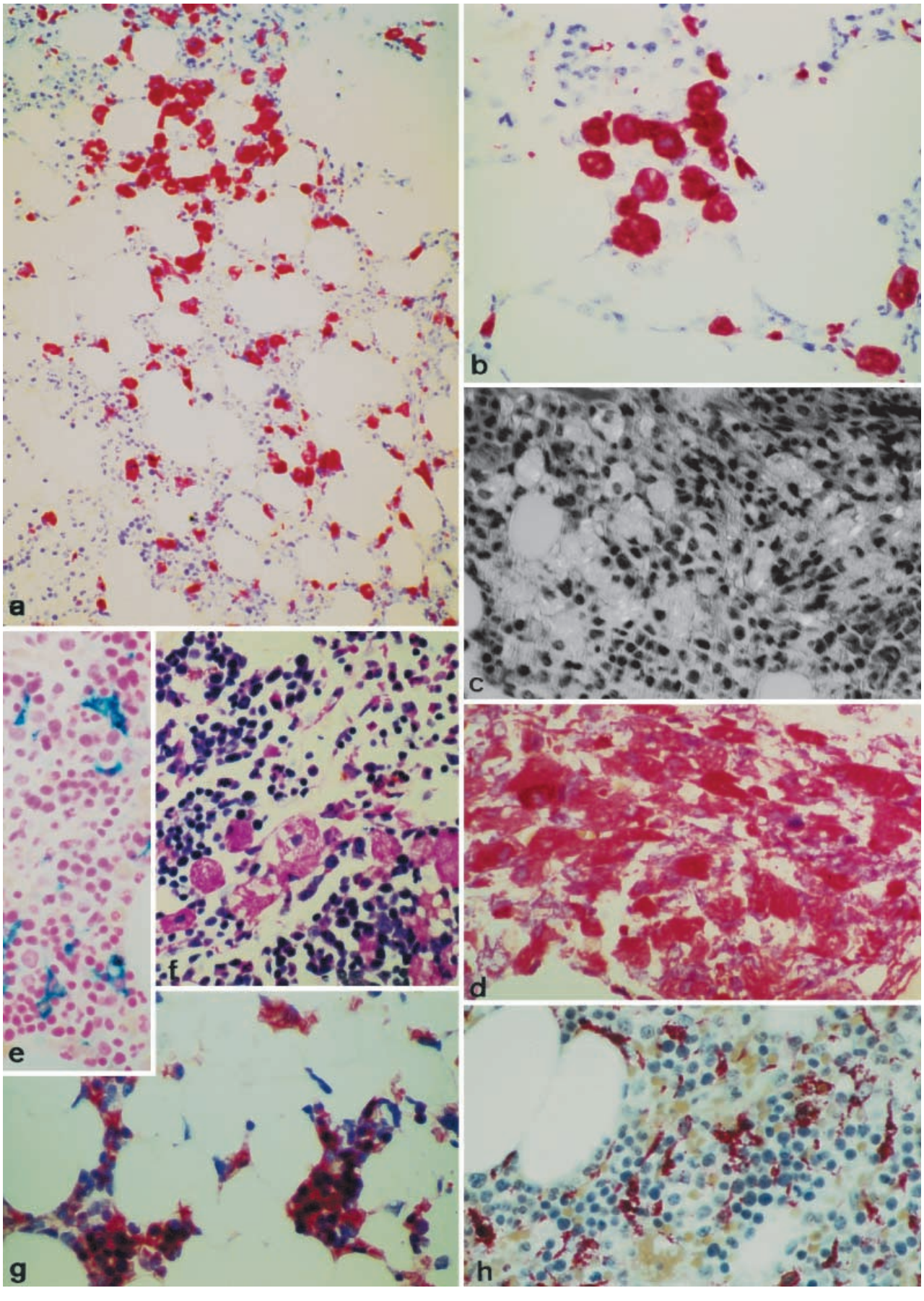
Endpoint	Before BMT (day 0)	After BMT				Normal bone marrow
	I	II	III	IV	V	
Intervals (days) range	1–120	0–30	31–45	46–119	120–365	–
Mean±SD	24±23	22±6	38±4	64±17	179±61	
No. of patients	126	25	50	55	18	25
Cellularity (%)	86±17	47±13	54±17	54±19	50±22	57±12
$CD68^+$ macrophages (mm^2)	358±100 (407±120)	360±125 (385±264)	329±122 (489±167)	322±107 (531±192)	322±177 (555±188)	372±86 (785±183)
BSA-I^+ macrophages (mm^2)	199±66 (234±78)	184±65 (193±146)	163±70 (245±101)	163±64 (265±118)	192±136 (306±150)	204±67 (490±108)
Pseudo-Gaucher cells (% present)	37.5	36.0	21.6	5.5	11.1	0
Iron-laden macrophages (% present)	19.3	76.0	98.0	92.6	24.4	85.7

failure of molecular-biological or cytogenetic investigations to reveal corresponding findings. Contrasting with their pretransplant scarcity and weak staining reaction, the fraction of iron-laden macrophages displayed not only an increase with coarse deposits of positive granules, but also a posttransplant prevalence in almost all patients (Fig. 2e). In comparison with the quantity of nucleated erythroid precursor cells (280±185/mm^2) in the pretransplant bone marrow samples (endpoint I, Table 1), there was a conspicuous reduction ($P<0.05$) in the postgraft specimens (184±150/mm^2). The fraction of BSA-I^+ macrophages, which regularly included the PGCs (Fig. 1c, d), exhibited a significant correlation ($r=0.424$, $P<0.01$) with regenerating erythropoiesis calculated per haematopoietic area in the posttransplant bone marrow shortly after BMT (endpoint II). This close spatial relationship between macrophages and reconstituting erythropoietic islets was demonstrable not only for the $CD68^+$ macrophages, but also for the PGCs (Fig. 2f–h).

Discussion

In the context of our study it seems to be reasonable to assume that quantification of the $CD68^+$ and BSA-I^+ macrophage population in the posttransplant bone marrow specimens has to be carried out with reference to the normal haematopoiesis. Bearing this point in mind, a properly conducted evaluation should also take account of cellularity, and consequently calculations have to be focused on the areas containing haematopoietic tissue. When this postulate is taken into account, an incidence of macrophages is detectable in the posttransplant samples (Table 1) which in the first 2 months is about 40–50% lower than that in corresponding areas in the normal bone marrow [34]. This significant reduction in the amount of macrophages per total haematopoiesis in the postgraft samples probably has functional implications, particularly for the reconstitution of erythropoiesis. Amongst other ill-defined and mediator-stimulated functions this includes degradation of the expelled normoblastic nuclei, iron turnover, haemoglobin synthesis and erythropoietin production [25, 37, 38]. Because there is a significant decrease in the number of erythroid precursor cells following BMT, it is tempting to assume a mutual relationship. Moreover, as a sequel to intensively performed supportive transfusion therapy in the immediate posttransplant period and various disturbances of iron metabolism following myelo-ablative treatment, the conspicuous increase in iron-storing macrophages is understandable. Until now a conflict of opinion arises regarding so-called macrophage activation [1]. Evidence for an activation of α-D-galactosyl residues expressing (BSA-I^+) macrophages is rather circumstantial and entirely based on in vitro findings in rodents [1, 4, 16, 19]. Therefore, it is noteworthy that, as demonstrated in this study, not only were PGCs characterized by an identical carbohydrate-binding capacity, but the BSA-I^+ subset of macrophages revealed a significant correlation with regenerating erythropoiesis.

Although a wealth of data has accumulated in recent decades on the occurrence of PCGs in CML [2, 3, 7, 10, 15, 18, 20, 31, 32, 34], controversy and discussion still persist about their incidence, which has been reported to range between 20% and 70% [7]. Moreover, PCGs are not only observed in CML, but occasionally also in acute myeloid leukaemia (AML) and various reactive and congenital conditions [15], including thalassaemia [40] and dyserythropoietic anaemia type II [36]. Ultrastructural studies are in keeping with the finding that PGCs differ from the storage cells in Gaucher's disease by their microfibrillar constituents of the lysosomal matrix [21]. Crystal-storing macrophages in multiple myeloma or lymphoplasmacytoid lymphoma may adopt the appearance of Gaucher cells and have therefore occasionally been called PGCs [24, 28]. However, persuasive evidence has been produced indicating that, in contrast to the glucocerebroside-laden true Gaucher cells, these are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characterized by a deposition of hydrophobic proteins showing needle-like crystalline structures [26]. Acquired PGCs, blue-pigmented histiocytes and other varieties of compound lipid-storing cell categories are thought to result from an extensive granulocytic and/or erythrocytic breakdown with pronounced phagocytosis of cell debris [10, 15, 18, 20]. Electron microscopical findings support this, as they demonstrate the presence of stored glycolipid material (glucosyl ceramide) originating from ingested and degraded granulocytes in the PGCs [21]. The greatly expanded bulk of granulocytes in CML presents a mass of glycolipids, which the stimulated and competent mononuclear macrophage cell compartment is unable to process [17], although there is normal or even increased lysosomal enzyme activity. Inefficient erythropoiesis in thalassaemia results in an overproduction of catabolic substances probably derived from the glycolipid-rich membranes of the erythrocytes [40]. As in CML patients, an overload of glucocerebroside induces a corresponding enzyme activity, which again is not able to catabolize these substrates. As a consequence, it is feasible to distinguish true Gaucher cells from PGCs in the few patients suffering from both conditions [29]. Altogether, all types of storing macrophages appear to be virtually restricted to pathological states of haematopoiesis associated with an extremely highly enhanced turnover of granulo- and erythropoiesis [15].

Regarding the pre- and posttransplant occurrence of PGCs in the CML bone marrow several other points have to be addressed. First, a scrutinized histomorphological evaluation suggested that among features of marginal importance, these cells may serve as an invaluable aid to differentiate early stages of CML from leukaemic reactions [27]. Secondly, the presence of PGCs at a patient's presentation has repeatedly been reported to have a favourable impact on prognosis [2, 20, 32]. Furthermore, studies with proliferation markers point to the fact that resident bone marrow macrophages are nonreplicating "endcells" [35]. Their continuous repopulation depends entirely on the monocytes, which are derived either from the local bone marrow pool or from the monocytes circulating in the blood. For this reason, PGCs retrieved after BMT were generated from the engrafted monocyte compartment of the donor, judging by their progeny. In contrast to an anecdotal report on two patients [3], in our series we found that postgraft recurrence of PGCs did not indicate CML or a leukaemic relapse, since cytogenetic and molecular-biological data failed to disclose a clonally transformed cell population. Considering the presence of PGCs in the pretreatment bone marrow in CML, the temporal reappearance of these specific cells in a significant number of patients immediately after BMT warrants explanation. In line with findings in a variety of haematopoietic conditions [15, 24, 28, 36, 40], the development of posttransplant PGCs is thought to originate from the failure of the macrophages to catabolize the bulk of cell debris as a sequel to myelo-ablative therapy, despite normal functioning of their lysosomes. For this reason, the PGCs retrieved may be regarded as scavenger macrophages occurring most frequently in the first few weeks after BMT.

In conclusion, our morphometric and immunohistochemical analysis of the resident bone marrow macrophage compartment following allogeneic transplantation in CML reveals a temporal retrieval of PGCs in the recovering haematopoiesis. This is probably generated by an enforced removal of the cell debris after pretransplant myelo-ablative therapy (scavenger function). Moreover, the subset of α-D-galactosyl-expressing macrophages constitutes an activated fraction; PGCs were also characterized by an identical carbohydrate-binding capacity. Their significant correlation with the number of erythroid precursors in the regenerating haematopoiesis shortly after BMT implicates an important functional aspect.

◀ **Fig. 2a–h** Macrophages and regenerating haematopoiesis in the CML bone marrow after BMT. **a** Loose clusters of large $CD68^+$ macrophages, **b** some of them apparently consistent with pseudo-Gaucher cells (PGCs). **c** Positive birefringence following polarization after Giemsa staining characterizes dense clusters of PGCs, which also show a distinctive BSA-I reactivity (**d**). **e** Iron-laden macrophages with coarse deposits. **f** PGCs among nests of reconstituting erythropoiesis compatible with successful engraftment. **g** Delayed engraftment with only small islets of immunostained erythroid precursors. **h** Close contact of centrally localized stellate $CD68^+$ macrophages in regenerated and confluent erythropoietic islets. **a** CD68, ×170; **b** CD68, ×370; **c** polarization after Giemsa stain, ×370; **d** BSA-I, ×370; **e** Perls' reaction, ×370; **f** PAS, ×370; **g** Ret40f, ×370; **h** CD68, ×370

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