Natural killer (NK) cells are phenotypically defined as lymphocytes expressing the antigens CD56 and mostly CD16 (FcγRIII), but lacking CD3. A small CD3− CD16− CD56+ NK cell subset has been described in normal individuals representing less than 2% of peripheral blood lymphocytes. We analyzed here patients for their reconstitution of the immune system 35% of these patients, 70 patients for their reconstitution of the immune system transplantation. In 35% of these patients, two different NK cell subsets, namely CD56dim and CD56bright, cells, were observed. The mean duration of these two subsets after transplant was 4 months. Sixty-five percent of the patients harvested an increased number of NK cells, but only the typical CD16+ CD56dim population. The CD56bright subpopulation represented a particular CD3− CD16− NK subset, with posttransplant frequencies up to 70% of all NK cells and 40% of peripheral blood lymphocytes, respectively. In contrast to normal CD56dim NK cells, CD56bright cells coexpressed the activation antigens p75 β-chain of interleukin-2 receptor (IL-2R), CD2R, and CD26, but were negative for CD16. NK and antibody-dependent cellular cytotoxicity activity of CD56bright cells was low compared with CD56dim NK cells. But using IL-2 and interferon γ, their cytotoxicity could be enhanced even more than in CD56dim lymphocytes. These different subsets may reflect distinct activation or differentiation steps of NK cells during reconstitution of the immune system. Their differential response to IL-2 may be of functional importance for posttransplant cytokine therapy.

Natural Killer (NK) cells are defined functionally by their ability to spontaneously lyse tumor cells or virally infected cells without major histocompatibility complex (MHC) restriction. From various studies they are assumed to play an important role after bone marrow transplantation (BMT) by mediating the graft-versus-leukemia effect. Phenotypically, NK cells are characterized by expression of the CD56 (NKH-1) antigen. This antigen represents the 140-Kd isoform of the neural cell adhesion molecule (N-CAM) molecule, and is in peripheral blood (PB), largely specific for NK cells. But it also can be detected on non-MHC-restricted cytotoxic lymphocytes and in vitro-activated T-cell clones. In addition, the majority of NK cells can be identified by monoclonal antibodies (MoAbs) against the CD16 (FcγRIII) antigen functionally responsible for the ADCC exerted by NK cells. By definition all NK cells are CD3− T-cell receptor (TCR) negative.

The lineage and ontogeny of NK cells is still a controversial issue. There are characteristics of T-cell lineage, such as the incomplete message of the β-chain of the TCR, CD2 expression, and use of the ζ-chain of the CD3 complex. On the other hand, antigens of the myeloid lineage, such as CD16 or CD11b, are expressed. Recently, a small subset of PB lymphocytes (PBL) (<2%) has been described in normal individuals expressing CD56 brightly that are also CD3− and CD16−. CD16− NK clones could be isolated from human thymus. The detection of this subset has led to new speculations regarding the in vivo differentiation of NK cells.

In the present study, the reconstitution of NK cells in patients after BMT was analyzed. In contrast to normal NK cells, the presence of two distinct NK cell subpopulations, namely, CD16+ CD56dim and CD16− CD56bright cells, could be detected. In addition, the different phenotypic and functional characteristics of these NK cell subsets are described.

MATERIALS AND METHODS

Patients. The characteristics of the patients examined are summarized in Table 1. All patients were analyzed for the phenotypic pattern of PBL weekly post-BMT.

Cell preparation. Heparinized blood samples were obtained from the patients as soon as lymphocytes were identified in the blood. The blood was diluted with an equal volume of Hank's Balanced Salt Solution and PB mononuclear cells (PBMC) were separated by centrifugation over a ficoll-hypaque gradient (Se-romed, Berlin, Germany). Nonadherent cells were obtained from PBMC by 1 hr of adherence on plastic culture dishes at 37°C. For some experiments, these cells were sorted using a FACStar Plus (Becton Dickinson, Heidelberg, Germany). Cells were stained with CD56 (Leu19 PE) MoAbs for 30 minutes at 4°C, washed three times, and sorted corresponding to the density of CD56 surface expression into CD56dim and CD56bright cells at a rate of 3,000 cells/s. When the cell counts were too low for FACStar sorting, lymphocytes were depleted of T cells by using CD3 MoAb (OKT3) and magnetic goat antimouse IgG beads (Dynabeads M450; Dynal, Oslo, Norway). Negative cells were depleted of either CD56 bright cells using p75 MoAb (Tu27) or CD56dim cells using CD16 MoAb (B73.1) and Dynabeads. For depletion, cells were incubated with the primary MoAb for 30 minutes at 4°C. After three washes, Dynabeads were added at a bead:cell ratio of 10:1. After 30 minutes of incubation on ice, cells were separated using a magnetic particle concentrator (Dynal) for 5 minutes. All purification steps were performed in RPMI 1640 containing 1% human AB serum.

MoAbs. The MoAbs used have been extensively characterized: CD2 (Leu2), CD3 (Leu4), CD8 (Leu5b), CD16 (Leu11b), CD20 (Leu10), CD56 (Leu19 PE), and CD25 were purchased from Becton Dickinson. CD3 (OKT3), CD4 (OKT4), and CD8 (OKT8) were from Ortho (Neckargemünd, Germany). CD2R (T11.3), CD26, and CD56 (NKH-1A) were generously provided by Drs S.F. Schlossman, E. Reinherz, and J. Ritz (Dana Farber Cancer Institute, Boston, MA). CD16 (B73.1) was a generous gift from B. Perussia (Thomas Jefferson University, Philadelphia, PA). The

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with a 1:5 solution of a commercial intravenous IgG (Intraglobin; Behringwerke, Marburg, Germany). Tu27 (anti-p75) was generously given by Dr K. Sugamura (Tohoku University School of Medicine, Sendai, Japan).

**Phenotypic analysis.** Phenotypic analysis was performed either by indirect immunofluorescence with fluorescein-conjugated goat anti-TCR2 antibody (BMA031) was a gift from Dr R. Kurrle (Behringwerke, Marburg, Germany). Tu27 (anti-p75) was generously given by Dr K. Sugamura (Tohoku University School of Medicine, Sendai, Japan).

**Phenotypic analysis.** Phenotypic analysis was performed either by indirect immunofluorescence with fluorescein-conjugated goat antihuman IgG (IgM-FITC, Dianova, Hamburg, Germany) or by direct immunofluorescence in two-color analysis using directly labeled antibodies. The staining procedure has been described in detail previously. Briefly, 1 to 3 × 10⁶ cells/well were incubated with murine MoAbs at an optimal dilution for 30 minutes. Non-specific binding was eliminated by mixing the samples with a 1:5 solution of a commercial intravenous IgG (Intraglobin; Biotest, Frankfurt, Germany). After three washes, direct immunofluorescence was analyzed. Samples for indirect staining were cultured after magnetic bead depletion for 24 hours in RPMI 1640 supplemented with 500 U/mL recombinant interleukin-2 (rIL-2; Cetus, Emeryville, CA) or 200 U/mL interferon γ (IFNγ; Biogen, Cambridge, MA) as described previously. Control cells were cultured without cytokines.

**RESULTS**

**CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells after BMT.** Phenotypic analysis of PBL after BMT showed an additional second NK cell population in about 35% of the patients. With an increase of NK cells, a CD56<sup>dim</sup> and a CD56<sup>bright</sup> subset were observed (Fig 1). The phenomon was equally distributed among autologous (7 of 20 [35%]) and allogeneic (18 of 50 [36%]) transplants, and female and male patients, respectively (Table 1). In the allogeneic transplant patients, the reconstituted NK cells were shown to be of donor origin. There was no obvious association of the appearance of this additional NK subset with any feature,
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CD56+ CD16+ NK cells. Post-BMT patients have a CD56+dim and CD56+bright NK cell population. The time course of CD56 and CD16 expression during 9 months after BMT in one representative patient is illustrated in the lower panel. For each sample, 10,000 cells were analyzed using a FACScan. Indirect immunofluorescence was performed as described in Materials and Methods.

including underlying disease, graft-versus-host disease (GVHD), viral infections, and course of reconstitution.

The CD56+ bright cells could be observed for a mean period of 17 weeks in each patient (Fig 1 and Table 1). The initial small proportion of CD56+ bright NK cells increased to a maximum of about 20% to 70% of all NK cells. This increase is also reflected in the absolute number of NK cells (Table 1). Finally, the CD56+ bright NK cell subset disappeared.

Phenotypic characteristics of the two distinct NK cell subgroups. Using two-color immunofluorescence, both NK cell subsets were characterized in more detail. Three main populations of CD56+ cells could be distinguished by comparison of the three panels in Fig 2 (A through C). The classical NK cell phenotype in the first subset was characterized as CD2+ CD3− CD16+ CD56+dim. The second NK cell subset was clearly different from the typical NK phenotype. These cells were CD2+ CD3− CD16− CD56+bright. A low proportion of CD56+dim cells coexpressing CD3 was not further studied because they were T cells by definition (Fig 2).

Activation antigens on CD56+dim and CD56+bright NK cells. When testing different activation antigens on NK cells, they were preferentially expressed by CD56+ bright cells. They were CD26+ CD2R+ HLA-DR+ p75+ (β-chain of IL-2 receptor [IL-2R]) (Fig 3). In contrast, the α-chain of the IL-2R (CD25) could not be detected on either of these subsets at any time. In regard to other lymphocyte antigens, similarities in both NK cell populations were observed. CD8 antigen expression could be detected in both NK cell subsets with varying percentages. The T- and NK-cell--
associated antigen CD2 was equally distributed in both NK cell subsets.

In Fig 4, the cell surface antigen expression of four different patients is summarized. The differences in expression of activation antigens become particularly apparent.

Cytosmears showed both NK subtypes to be large granular lymphocytes (LGL), confirming the additional NK subgroup as NK cells also by morphologic criteria with typical cytoplasmatic granules (data not shown).

**CD56^{bright} NK cells exhibit low cytotoxicity.** Freshly isolated PBMC and CD56^{dim} and CD56^{bright} cells were tested for their NK and ADCC activity. Cytotoxicity of unseparated lymphocytes against K562 and ADCC against antibody-treated L1210 showed in all patients normal values. But both NK cell types were functionally distinct when examined in detail using CD56^{dim} and CD56^{bright} cells after FACS sorting or magnetic bead enrichment. Freshly FACS-purified CD56^{dim} cells exhibited high NK killing and ADCC activity. It is evident that NK and ADCC killing of CD56^{bright} cells is significantly lower than that of CD56^{dim} NK cells (Fig 5A). On the other hand, after IL-2 or IFNγ activation, the relative increase of cytotoxicity in these CD56^{bright} cells is higher compared with the CD56^{dim} cells (Fig 5B and C).

**DISCUSSION**

For a number of years the CD56 or NKH-1 molecule has been shown to represent the most suitable surface antigen to identify PB NK cells. However, the functional nature of this antigen on NK cells is still a matter of discussion. Recently, two groups described a NK cell subset characterized by a high expression of CD56 antigen. They have observed these NK cells in normal donors representing only less than 2% of all PBL. We characterize here the CD16^- CD56^{bright} NK cell subset in comparison to normal CD16^+ CD56^{dim} NK cells during reconstitution after BMT (Figs 1 and 2). The reconstitution phase after BMT, cancer treatment using the rIL-2, and finally, T-deficient severe combined deficiencies (unpublished observations) represent clinical disease states in which high frequencies of NK cells
cells (up to 70% to 90%) can be observed. This high proportion of NK cells usually goes along with the presence of this particular CD56^bright NK subset. Therefore, these disease entities provide models for studying activation and differentiation of human NK cells in vivo.

Our phenotypic data show a much higher activation status of CD56^bright compared with CD56^dim cells (Figs 3 and 4). The CD16^CD56^bright cells all coexpress the p75 β-chain of the IL-2R, but do not express the high-affinity IL-2R. This finding is consistent with in vitro IL-2-activated NK cells in tumor patients. In earlier studies it has been shown that the p75 expression on these CD56^bright cells is responsible for IL-2-dependent induction of proliferation and in vitro activation of cytotoxicity. Therefore, it must be concluded that chronic exposure to IL-2 in vivo may be responsible for the lack of the CD25 IL-2R. In addition, the CD26 activation antigen is expressed on all CD56^bright cells. The CD56^bright NK cell subset post-BMT, in contrast to normal NK cells, does not exhibit the CD16 or FcγRIII structure. This phenotype has also been detected for a small CD56^bright subset in normals. It is consistent with the functional observation that these cells can hardly perform any ADCC (Fig 5A). CD2R is present on 50% of these cells. In contrast, none of these activation structures, with exception of very little p75, can be detected on CD16^CD56^dim NK cells. The cytotoxic function of CD56^bright cells appears to be significantly decreased compared with the CD56^dim NK cells. But after in vitro activation using IL-2 and IFNγ, cytotoxicity enhancement can be observed for both NK subsets, relatively stronger for the CD56^bright cells. Interestingly, the finding of two different CD56^+ cell subsets is a transient one (Fig 1). During reconstitution it can be detected during a mean period of about 4 months. At this point, it can only be speculated that most likely the early reconstitution of the lymphoid system in the presence of high cytokine concentrations, in particular IL-2, might enhance this CD56^bright NK subset. The lack of CD16 expression and diminished NK cell activity suggest the hypothesis that the CD56^bright cells represent an early NK progenitor cell subset that might differentiate into mature NK cells. Alternatively, the CD16^CD56^bright NK cells might be the result of a maximal in vivo activation of NK cells after BMT. This activation may induce an increased CD56 and p75 antigen expression, and at the same time a loss of FcγRIII or CD16 antigen.

Therefore, further in vitro activation and molecular studies will have to clarify the functional role of the two NK cell subsets expressing different CD56 antigen density. This knowledge may be particularly important when using IL-2 treatment for patients after BMT.

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CD16- CD56+ natural killer cells after bone marrow transplantation

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