Brief Definitive Report

A Novel 80-kD Cell Surface Structure Identifies Human Circulating Lymphocytes with Natural Killer Activity

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Summary

Human lymphocytes with natural killer (NK) activity, including most activated γ/δ T lymphocytes, recognize and lyse tumor target cells without requiring recognition of major histocompatibility complex antigen. However, unlike γ/δ T lymphocytes, NK cells do not express CD3/T cell receptor (TCR) molecules, and the receptors involved in cell-mediated cytotoxicity are unknown. To further delineate circulating NK cells, we developed monoclonal antibodies (mAbs) against the human NK leukemia YT2C2. We report the isolation of a mAb termed BY55, recognizing at the cell surface a novel 80-kD protein with restricted expression. In addition to the immunizing cell line, this mAb binds to circulating NK cells, γ/δ + cells, and a minor subset of α/β + T lymphocytes. Expression of the BY55 mAb-reactive epitope/molecule is regulated by activation, as short-term culture of peripheral blood lymphocytes (PBL) with phorbol ester induced its downmodulation. Furthermore, BY55 mAb reactivity was found neither with the NK nor with the TCR α/β + and γ/δ + clones tested. Biochemical studies as well as phenotypic analysis revealed that this structure is different from all previously identified molecules on the lymphocyte cell surface. Interestingly, we found that BY55 + cells exert most NK activity obtained with fresh circulating lymphocytes. We report that within fresh E rosette-positive PBL only a subset of the CD16 +, CD56 +, and CD57 + cells coexpressed BY55 molecule, indicating that BY55 mAb defines a unique subset mediating NK activity of circulating PBL.

NK cells are circulating lymphocytes found within the large granular lymphocyte population (1, 2) and constitute, with CTL (3), the major cytotoxic effector lymphoid elements of the immune system. The characteristic surface markers expressed by NK cells are CD56 (4-6), CD57 (4, 5), CD16 (7-10), CD11b (11), and CD11c (11, 12). Most circulating cells with NK activity express the CD2 molecule but are distinct from the T lineage, as they do not express CD3 and do not rearrange any of the TCR genes (13, 14). In addition, NK cells mediate cytotoxicity without requiring recognition of MHC molecules on target cells. However, most activated TCR γ/δ lymphocytes (15-18), and under certain circumstances some TCR α/β cloned lymphocytes (19), can also mediate MHC-unrestricted cytotoxicity. The NK cell receptor(s) participating in target recognition remain(s) largely unknown despite extensive studies (20-22).

In an attempt to define NK cell-specific surface structures, we characterized a mAb obtained by repeated immunization with YT2C2, a human cell line with NK functional characteristics. This cell line was selected for intermediate affinity binding of 125I-IL-2 with the IL-2R p75 subunit (23). In the present study, we report the isolation of a mAb, termed BY55, reacting at the cell surface with a novel 80-kD protein structure. BY55 mAb reactivity is observed with 15-25% of circulating lymphocytes. Two-color immunofluorescence staining of fresh E rosette-positive (E +) PBL obtained from normal individuals revealed that a sizeable proportion of CD3-negative and TCR γ/δ + lymphocytes are stained by BY55 mAb, whereas, in contrast, a minor subset of CD8 + α/β + T lymphocytes expressed BY55-reactive molecule. Moreover, activated PBL and TCR α/β or γ/δ T cell clones failed to express the BY55 mAb-reactive epitope/molecule. To address the question as to whether the BY55 + circulating lymphocytes could be responsible for NK activity, negative (complement-dependent depletion) and positive (sorted population) selections of the BY55 mAb-reactive population were performed. Results indicate that NK activity is mediated by BY55 + circulating lymphocytes. As we found that the BY55 mAb defined the circulating NK cell population, partly distinct from those defined by CD56, CD57, or CD16 mAb,
we conclude that BY55 + cells represent the subset of circulating competent cytolyltic effectors in NK cells.

Materials and Methods

Production of BY55 mAb This mAb of IgM isotype was obtained by immunizing BALB/c mice with the human NK cell line, YT2C2, generously provided by Dr. S. Chouaib (IGR, Villejuif, France). Cell fusions were performed as previously described (24, 25). The initial screening by indirect immunofluorescence and flow cytometry using a FACStar® (Becton Dickinson & Co., Mountain View, CA) retained all hybridoma culture supernatants reacting with the immunizing cells, but not with the T cell clone JF1 (19) and an EBV-transformed B cell line. Ascites were purified by precipitating IgM proteins in dialysis with distilled water followed by gel filtration chromatography.

mAbs. CD3-FITC, CD4-FITC, CD8-FITC, CD19-FITC, CD57-FITC, CD16, and CD56 were purchased from Immunotech (Luminy, France). TCR a/ß was kindly provided by Dr. M. Brenner and the anti-TCR a/ß BMA031 was a gift from Dr. A. Musielli (Behring, Rueil, France). ßTCS1 was purchased from T Cell Sciences (Cambridge, MA). The anti-Vß2, BB3 mAb was kind gift of Dr. A. Moretta. The mAbs CD2 (CD2x11 and D66), CD3 (CD3x3), CD4 (0516), CD8 (L533), CD25 (BC96), and BB18 were produced and purified in our laboratory.

Isolation of Cell Populations. Human PBMC were prepared by Ficoll-Isoaque density gradient centrifugation. The unfraccionated population was separated into E rosette-negative (E-) and E rosette-positive (E+) populations by rosetting with 5% SRBC as previously described (24). BY55 + E- PBL were obtained by further treatment of E+ cells with BY55 mAb plus rabbit complement. For the isolation of BY55+ and BY55- cell populations, fresh PBL were stained by indirect immunofluorescence with BY55 mAb and sorted into BY55 + and BY55 - fractions using a FACStar microfluorometer (Becton Dickinson & Co.). Sorted populations were collected as described elsewhere (26), were washed twice, and tested as effector cells in cytotoxic assays. Trypan blue exclusion was performed on the unsorted population and sorted populations, and the viability was always >90%.

Immunofluorescence Assays. Indirect immunofluorescence assays were performed using a FITC-conjugated anti-mouse Ig. Fluorescence was read using a FACStar® microfluorometer (Becton Dickinson & Co.). For two-color immunofluorescence experiments, cells were simultaneously incubated for 30 min with a FITC-conjugated mAb and biotin-conjugated BY55 mAb. The cells were then washed before an appropriate amount of PE-conjugated streptavidin was added. Alternatively, an indirect assay was done with some uncoupled IgG mAb using an FITC anti-mouse IgG. After final washes, the cells were resuspended in 0.3 ml of cold 1% formalin solution in PBS. Purified BY55 antibodies were biotin labeled using a standard protocol (26). Fluorescence emissions of FITC and PE were distinguished using 530 ± 15 and 575 ± 12.5 nm bandpass filters, respectively. The overlap in FITC and PE fluorescence emissions was corrected by using an electronic compensation network.

PMA-Induced Modulation of Cell Surface Expression. PBMC were incubated with PMA (Sigma Chemical Co., St. Louis, MO) at 50 ng/ml in culture medium containing 10% NHS, at 37°C, for indicated times. After extensive washing, cells were stained for immunofluorescence using biotinylated mAbs.

Labeling and Immunoprecipitations of Cell Surface Proteins. YT2C2 cells were 125I surface radiolabeled using the lactoperoxidase method as previously described (25). Aliquots of precleared lysates were immunoprecipitated with either rabbit anti-mouse IgM Ab coupled to protein A-Sepharose beads as negative control, or with the same immunosorbant complex previously coupled with BY55 mAb. Immunoprecipitations, SDS-PAGE analysis, and autoradiography were performed as described (25).

Cytotoxic Assays. Cytotoxicity assays were performed according to a standard 51Cr release method (19, 26). All experiments were performed with fresh effector cells. Target cells were the NK-sensitive cell line, K562. Assays at various E/T ratios with 5 x 103 51Cr-labeled target cells/well were carried out in triplicate, using 96-well V-bottomed microtiter plates. The percentage of lysis was determined as previously described (19, 26).

Results

BY55 mAb Identifies a Unique CD2+ Lymphocyte Subset. BY55 is an IgM mAb. Extensive phenotypic analysis revealed that, in addition to the immunizing cell YT2C2 and its parental cell line YT, BY55 mAb only stained a subset of 10-25% of E- PBL (20 individuals tested). Specifically, no BY55 mAb staining could be detected among PHA- or IL-2-activated E+ PBL, granulocytes, monocytes, platelets, red blood cells, and bone marrow cells. In addition, BY55 mAb did not bind to 16 different leukemic cell lines, 5 EBV-transformed B cell lines, and 20 rIL-2-dependent CD3+ or CD3- cell clones tested (data not shown). Next, we determined whether this expression was restricted to any particular subset of peripheral blood CD2+ cells by two-color immunofluorescence staining of E+ PBL derived from normal individuals. The results obtained with one representative donor's PBL with 23% of BY55+ cells are shown in Fig. 1. Among BY55+ cells, 50% expressed TCR a/ß, 25% expressed TCR ß/ß, while the remaining 25% were unreactive with anti-TCR or CD3 mAb. In addition, a minor proportion (2-4%) of the BY55+ cells were CD4+, while 50% coexpressed CD8 molecules. Further, within BY55+ TCR ß/ß cells, an equal proportion reacted with BB3 or ßTCS1 mAb. Conversely, a majority of CD2+ CD3+ cells were labeled by BY55 mAb. Thus, while BY55 mAb delineated a subset of cells clearly different from all well-identified T cell subsets, it was important to determine whether it was expressed by CD2+ cells known to exhibit NK activity.

Differential Distribution of the NK-associated Molecules CD16, CD56, or CD57 and BY55 Monoclonal-reactive Molecule. Therefore, we first compared BY55 expression with the NK-associated molecules CD16, CD56, and CD57. Table 1 shows the median percentages obtained in three independent two-color fluorescence experiments. When E+ PBL were stained simultaneously with BY55 and CD16 mAb, only one-half of the CD16+ cells and 40% of the BY55+ cells coexpressed both markers. Moreover, whereas two-thirds of CD56+ cells coexpressed BY55 molecules, only one-third of the lymphocytes coexpressed CD57 and BY55 molecules. Therefore, expression of the BY55 surface antigen appears to define a circulating lymphocyte subset clearly different from that previously defined as NK cells by CD16, CD56, or CD57 mAbs.

Downmodulation of BY55 Molecule after PMA Activation. Because BY55 mAb failed to stain any short- and long-term
activated lymphocytes irrespective of their phenotypes, we investigated whether a potent inducer of lymphocyte stimulation such as PMA was capable of modifying BY55 expression. A 24-h incubation of E+ PBL (containing 28% of BY55+ cells) with PMA at 50 ng/ml resulted in an almost complete disappearance of BY55+ cells as shown in Fig. 2. The decrease in BY55+ cells is time dependent and is observed in the presence of PMA as early as 15 min. As control the staining of BB18 mAb was simultaneously analyzed and, as expected, the reexpression of BB18 molecule was observed at 24 h (24). It should be noted that reexpression of BY55

Table 1. Percentage of E+ PBL Coexpressing CD16, CD56, or CD57 Molecule with BY55 mAb-reactive Antigen

| Cell reactivity | CD16+ | CD16- | CD56+ | CD56- | CD57+ | CD57- |
|----------------|-------|-------|-------|-------|-------|-------|
| BY55+          | 6     | 9     | 10    | 7     | 9     | 7     |
| BY55-          | 6     | 79    | 5     | 78    | 23    | 61    |

Two-color immunofluorescence analysis of resting E+ PBL was performed as described in Materials and Methods. Results are the mean percentage of three independent experiments.
molecule was not observed even after 3 d of culture (data not shown).

**BY55 mAb Identifies a Novel 80-kD Molecule.** To confirm that BY55 mAb–reactive surface molecule was different from all previously defined T and NK cell surface structures, we performed the biochemical identification of the surface molecule recognized by BY55 mAb. YT2C2 cells were externally radiolabeled by the lactoperoxidase method and lysed. BY55 immunoprecipitates obtained from these labeled cell lysates were next analyzed by SDS-PAGE. Autoradiography shown in Fig. 3 indicated, in the BY55 mAb immunoprecipitate, a predominant broad band migrating with an approximate molecular mass of 80 kD, under both nonreducing and reducing conditions. The immunoprecipitates obtained from a lysate of peripheral blood population presented identical characteristics (data not shown).

**NK Activity of Circulating Lymphocytes Is Restricted to the BY55 + Subset.** As most CD2+CD3− cells were stained by BY55 mAb, it was important to determine the cytotoxic activity of these BY55 + cells. First, we looked at whether NK activity of E + PBL was abolished after depletion in BY55 + cells by C-dependent lysis. Fig. 4 A shows the representative results obtained with one normal donor. Similar results were obtained with five different individuals. Fresh E + PBL were treated or sham treated with BY55 mAb and complement before tested as effector cells against the NK-sensitive target cell K562 at different E/T ratios. Whereas no difference in terms of killer activity was observed in untreated and sham-treated E + PBL (data not shown), the BY55− E + PBL failed to exhibit any significant NK activity even at an E/T ratio of 100:1. Thus, BY55 + PBL were necessary for NK activity against standard target cells. To determine whether BY55 + circulating lymphocytes represented the effector of NK activity, PBL were labeled with BY55 and sorted into BY55 + cells and BY55− cells using a FACS®. The results from a representative experiment is presented in Fig. 4 B. Whole PBL and BY55 mAb–labeled PBL of this donor exhibited a strong NK activity. Interestingly, and as expected from the later experiments, BY55− cell population was deprived of NK activity. Moreover, BY55 + population was greatly enriched in cells mediating NK activity, as shown by a higher percentage of specific 51Cr release at each E/T ratio. It has to be noted that treatment of PBL by BY55 mAb did not modify NK activity.

**Discussion**

In the present report we described a novel 80-kD molecule exclusively expressed by a subset of circulating human CD2+ lymphocytes responsible for killer activity against the standard NK-sensitive target cells. We found that activation of E + PBL rapidly resulted in the disappearance of the molecule/epitope detected by the BY55 mAb at the cell surface.
Thus, it appears that the BY55 mAb-reactive molecule corresponds to a cell surface differentiation antigen delineating a functional circulating lymphocyte population.

Analysis of BY55 distribution on E+ PBL demonstrated that the BY55+ subset was mostly confined to the CD3– and to TCR γ/δ+ lymphocytes. Further, double fluorescence analysis showed that a subset of CD16+, CD56+, or CD57+ cells coexpressed BY55-reactive molecule. These molecules are the prototypic antigens expressed by NK cells (27–29), and as we found that removal of BY55+ cells in E+ PBL resulted in the abolition of the NK activity, the BY55 mAb appears to define the active subset of E+ PBL with NK activity. This finding was further confirmed and extended to PBL, with the observation that positively selected BY55+ circulating lymphocytes exhibited highly efficient NK activity. Insignificant killer activity was found with the sorted BY55– population. In contrast to CD16 (FcRIII), a molecule expressed on NK cells, neutrophils and activated macrophages (30), and to CD56 (isoform of NCAM) (31), the expression of the BY55 molecule is restricted to a subset of lymphoid cells containing mainly circulating CD2+ CD3– and CD2– γ/δ+ cells. A mAb termed anti-Kp43, recognizing a 43-kD disulfide-linked dimer, has been reported with a similar pattern of reactivity (32, 33). It was selected for its ability to inhibit IL-2-dependent proliferation. In contrast to BY55, anti-Kp43 reacts with cultured NK cells and PHA-activated PBL, stimulates IL-2-induced proliferative responses, and induces MHC-unrestricted cytotoxicity. Nakamura et al. (34) reported two mAbs, YTA-1 and YTA-2, raised against YT cells. These mAbs recognize a 75-kD protein selectively expressed by large granular lymphocytes and all peripheral blood T cells. Interestingly, it was found that the YT-1/2 mAb were mitogenic for freshly isolated PBMC and could induce CD25 expression. As we were unable to find such agonistic properties of BY55, we exclude the possibility that the YT-1/2 mAb recognizes the BY55 molecule. Another mAb termed GL183 (35) identifies a subset of CD16+ NK cells. However, it differs from BY55 in its tissue reactivity and in its functional properties. Moreover, apparent molecular masses of the structures recognized by BY55 and GL183 are not identical.

Finally, it seems unlikely that BY55 reacts with IL-2R75 as BY55 does not inhibit IL-2-induced proliferation and IL-2-induced cytotoxic activity of NK cells. Comparative phenotypic analysis with an anti-IL-2R75, TU27 (36), revealed that TU27, but not BY55, stained IL-2-dependent T cell clones.

The fact that the expression of BY55 appears to be restricted to NK and γ/δ+ circulating cells raised the possibility that these populations share phenotypic characteristics in addition to their non-MHC-restricted killer activity (18). Further studies are underway to determine whether the BY55 molecule is preferentially expressed by the CD8+ or the CD8– cells in the TCR γ/δ+ and in the CD3+ lymphocyte subsets. As BY55 cell surface expression is associated with a high lytic activity, experiments in progress should determine whether the small subset of circulating CD3+CD8+BY55+ cells exclusively mediates CTL activity.

In conclusion, BY55 mAb, which stains a minor CD2+ subset, constitutes a unique tool to identify all circulating NK lymphocytes. Because the expression of this molecule is restricted to circulating uncultured lymphocytes, it represents a new marker of the differentiation/activation state of the NK cell subset (3). This probe will contribute to a better understanding of the immunobiology of circulating NK effector lymphocytes and to delineation of their participation in inflammatory disorders.

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