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Toru Abo, University of Alabama at Birmingham
Max Cooper, Emory University
Charles M. Balch, University of Alabama at Birmingham

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POSTNATURAL EXPANSION OF THE NATURAL KILLER AND KILLER CELL POPULATION IN HUMANS IDENTIFIED BY THE MONOCLONAL HNK-1 ANTIBODY*

BY TORU ABO, MAX D. COOPER, AND CHARLES M. BALCH

From the Cellular Immunology Unit of the Tumor Institute, Department of Surgery and Microbiology, and the Veterans Hospital Medical Center, University of Alabama in Birmingham, Birmingham, Alabama 35294

Natural killer (NK) cells capable of spontaneous lysis of tumor cells, and killer (K) cells that can lyse antibody-coated target cells have been demonstrated in many species (1-3). Much attention has been focused on these cells because of their possible role in the elimination of tumors and virus-infected cells (1). Although NK and K cells have been characterized as “non-T, non-B cells” because they are not generated in the thymus and do not express the usual array of T and B cell surface markers, their cell lineage remains an enigma. In humans, NK activity has been associated with a population of large granular lymphocytes (4, 5). NK cells are largely acquired after birth in rodents and pigs, but limited and conflicting information is available with regard to their ontogeny in humans (4, 6-9). In the earlier studies NK and K cells were defined primarily by their functional properties.

We have recently produced a monoclonal IgM antibody (HNK-1) that identifies a discrete population of lymphoid cells in humans with NK and K cell activities (10). In the experiments reported here, HNK-1 antibody was used to enumerate NK and K cells in human blood as a function of age and sex; NK and K cell activities were examined in parallel.

Materials and Methods

Cell Preparation. Mononuclear cells from heparinized peripheral blood of neonates and healthy adult donors were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) density gradient (10). The cells were washed three times with RPMI 1640 with 10% heat-inactivated fetal calf serum and 50 μg/ml gentamycin and suspended in this medium.

HNK-1 Monoclonal Antibody. HNK-1 is an IgM monoclonal antibody produced against a membrane extract of a human lymphoblastoid cell line (HSB-2) as previously described (10). This reagent will be available through the Becton, Dickinson & Co., Mountain View, Calif., and the American Type Culture Collection, Rockville, Md.

Immunofluorescence Assays. Cells reactive with a monoclonal antibody, HNK-1, were enumerated by an indirect immunofluorescence assay (10). The HNK-1 antibody was used at a concentration of 10 μg/ml for detection of both surface and cytoplasmic expression of HNK-1 antigen. Secondary antibodies were fluorescein isothiocyanate-conjugated (FITC) and tetramethylrhodamine isothiocyanate-conjugated (RITC) goat anti-mouse μ chain antibody (generously supplied by Dr. W. E. Gathing, University of Alabama in Birmingham) (11). The procedure for staining was performed as described elsewhere (10, 11).

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Age-related increase of HNK-1<sup>+</sup> cells in peripheral blood of healthy donors. The frequency of HNK-1<sup>+</sup> cells among mononuclear cells was determined in blood samples from 54 males (□) and 58 females (○) ranging in age from the day of birth to 88 yr. HNK-1<sup>+</sup> cells were enumerated by indirect membrane immunofluorescence using HNK-1 monoclonal IgM antibody and FITC-anti-mouse μ.

Natural Killer Assay. NK cell function was examined by a 51Cr-specific release assay using K562 target cells as described previously (10). Briefly, 100 μl of labeled 10⁴ K562 was incubated with 100 μl of effector cells at ratios from 1:1.25 to 1:20 for 6 h at 37°C in a humidified atmosphere containing 5% CO₂ in air. The percentage of 51Cr-specific release = 2S/M × 100, where S is the count of 100 μl of supernatant and M is the average count of maximum release from the labeled target cells. Percentages of spontaneous release without effector cells were <5.0%. All samples were assayed in triplicate cultures.

Results and Discussion

The monoclonal HNK-1 antibody was previously shown to react selectively with a discrete subpopulation of lymphoid cells with NK and K cell activities (10). Approximately 90% of the HNK-1<sup>+</sup> cells express surface receptors for IgG antibodies and contain azurophilic granules in their neutrophilic cytoplasm; some bind sheep erythrocytes to form rosettes, whereas others do not.

HNK-1<sup>+</sup> cells were enumerated in a group of 112 normal subjects who ranged in age from the day of birth to 88 yr. We found a highly significant correlation between age and HNK-1 antigen expression (r = 0.78, P < 0.0001). Among mononuclear cells in blood, the percentage of HNK-1<sup>+</sup> cells was only 0.5 ± 0.3% in 13 newborns (Fig. 1). This proportion increased with age so that HNK-1<sup>+</sup> cells comprised 5.1 ± 3.5% of circulating mononuclear cells in 21 children <15, 12.3 ± 5.1% in 29 individuals aged 15-30, and 22.2 ± 9.6% in 49 adults >30 yr old.

When the relationship between HNK-1<sup>+</sup> cell frequencies and NK cell functional activity was examined, an increased proportion of HNK-1<sup>+</sup> cells was found to correspond with an increased level of NK cell activity (Fig. 2). This correlation was most striking when comparing groups of newborns, children, and young adults. Even among older individuals, such as those in the 6th and 7th decade, a relationship between the proportion of HNK-1<sup>+</sup> cells and NK cell activity was still apparent, although the functional efficiency of NK cells appeared to be optimal in young adults (Fig. 2). These same relationships of antigen expression and function were also observed in a K cell lytic assay using sensitized chicken erythrocytes as the target and nylon wool nonadherent lymphocytes (to eliminate macrophages) as the effector cells (data not shown).
FIG. 2. Relation of HNK-1⁺ cell frequencies, functional NK cell activity, and age.

FIG. 3. Two-color immunofluorescent analysis of HNK-1 antigen expression on the surface membrane and in the cytoplasm of adult blood mononuclear cells. (a) Surface membrane expression of HNK-1 antigen (fluorescein stain) seen under incubation conditions favoring redistribution of cross-linked surface receptors (cells A, B, and C). (b) Cytoplasmic HNK-1 expression (rhodamine stain) observed in cells A and B but not in cell C (× 600).
In addition to HNK-1 expression on the surface membrane, we found a prominent cytoplasmic expression of HNK-1 antigen in some but not all cells (Fig. 3). Conversely, all of the cells with visible cytoplasmic expression of HNK-1 bore detectable HNK-1 antigen on their surface. The cytoplasmic HNK-1 expression was most prominent in adult blood lymphocytes. In eight adults, a mean of 51% of the surface HNK-1+ cells were cytoplasmic HNK-1+, whereas cytoplasmic expression was detectable in only 6% of HNK-1+ cells in blood samples (n = 6) from newborns and young children. We also observed that few surface HNK-1+ cells in adult bone marrow samples (n = 8) expressed cytoplasmic HNK-1 (mean, 18% positive). These observations suggest that cytoplasmic accumulation of HNK-1 molecules normally occurs in more mature cells of this lineage. Elucidation of the biologic significance of this awaits molecular and functional definition of the HNK-1 molecule.

The age-related changes that we have defined in the postnatal expansion of human NK and K cells are complementary to those previously observed in animals of other species. NK cell activity is not demonstrable during the first 2 wk of life in mice and rats (1, 2, 12); it begins to appear between 2 and 3 wk of age, then increases to maximum levels between the 5th and 8th wk. A delayed appearance of NK cell function has also been noted in pigs (3). In a previous study in humans, no clear relationship between NK and K cell functional activity and age was noted among individuals 20 yr of age or older (6, 7). Other investigators, using NK or K cell functional assays, have obtained results suggestive of a lower level of NK and K cell activities in blood cells from children compared with adults (4, 8, 9). The present results clearly show this to be the case, and suggest that the basis for the relative deficiency of NK and K cell activities in young humans is due to a numerical deficit of the cells with NK and K activities. In support of this conclusion, we observed in newborn blood a low frequency (<1.2% of mononuclear cells) of medium-sized lymphocytes with azurophilic granules using the May-Gr"{u}nwald-Giemsa stain. Although there is a correlation between NK function and HNK-1 antigen expression, there appears to be comparatively higher killing activity in cord blood (13, 14) than expected from the result of fewer proportion of HNK-1+ cells. In the functional assay using K562, cells other than NK cells may act as effector cells in cord blood, because analogues of NK cells have recently been associated with the killing of K562 (15). This issue is under investigation by cell sorting analysis using HNK-1 antibody.

With increasing age, an inverse relationship with NK function has been demonstrated in mice, with functional NK cell activity declining progressively beyond 12 wk of age (1, 2). Although we did not observe such a prominent age-related decrease in the level of NK function in humans, there was a broader range of HNK-1+ cell levels in older individuals (Fig. 1), and efficient NK activity was not always correlated with a higher frequency of HNK-1+ cells (Fig. 2). This raises the possibility that a dissociation between expression of HNK-1 antigen and NK cell function might exist in some elderly individuals. It is also conceivable that human NK cells are HNK-1 positive (10) but not all HNK-1+ cells are associated with killing of a particular target cell, because earlier studies using single cell agarose NK assay (16) and plaque K cell assay (17) indicated that only 1–5% of mononuclear cells were responsible for the above activities.

In addition to the correlation with age, we noted a correlation of HNK-1+ cell levels with the sex of the donor. A significantly higher proportion of HNK-1+ cells
was observed among 54 males than among 58 females (16.9 vs. 13.1%, $P < 0.05$) whose ages were comparable (mean ages: 30.2 vs. 32.1 yr). The slightly higher level of HNK-1$^+$ cells in males is in agreement with the findings of other investigators (4, 18, 19), who noted a lower level of NK or K cell function in females. It thus appears that sex hormones may influence the development of NK cells in humans. Such a hypothesis is supported by the demonstration that estrogen administration blocks maturation of NK cells in mouse bone marrow, although a nonphysiological dose of estrogen was administered in the study (20).

Although we have consistently observed HNK-1$^+$ cells in 15–17-wk fetuses (0.05–0.2% of cells in liver, spleen, and thymus), relatively few HNK-1$^+$ cells appear to enter the circulation by birth. This retarded developmental pattern, while as yet unexplained, may be unique for NK and K cells among blood cell types, since the proportions of T and B lymphocytes, monocytes, and granulocytes have already reached adult levels at birth.

**Summary**

Human natural killer (NK) and killer (K) cells were directly enumerated using a monoclonal antibody (HNK-1) and an immunofluorescence assay. The frequency of cells bearing surface HNK-1 antigen was very low in the newborn (<1.0%) and increased progressively through childhood and into adult life. This was correlated with an age-related increase in functional NK and K cell activities. Males had a slightly higher proportion of HNK-1$^+$ cells than females. In addition to HNK-1 expression on the surface membrane, a prominent cytoplasmic expression of HNK-1 antigen was found in some but not all surface HNK-1$^+$ cells. The cytoplasmic accumulation of HNK-1 molecules appeared to occur in more mature cells of this lineage.

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