Brief Definitive Report

INVOLVEMENT OF CD56 (NKH-1/Leu-19 ANTIGEN) AS AN ADHESION MOLECULE IN NATURAL KILLER-TARGET CELL INTERACTION

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The CD56 differentiation antigen, recognized by anti-Leu-19 and NKH-1 mAbs, is a 200–220-kD glycoprotein that is expressed predominantly on human NK cells and a minor subset of T lymphocytes mediating MHC-unrestricted cytotoxicity (1–4). A growing body of evidence has demonstrated that surface differentiation antigens expressed on functionally distinct lymphocyte subpopulations are implicated in their specific functions, however, the functional role of CD56 in NK cytotoxicity has not yet been determined.

Recently, it has been revealed that CD56 is an isoform of the neural cell adhesion molecule (N-CAM) (5), which is expressed on neural and muscle tissues and involved in homotypic adhesive interactions (6). This finding suggests that CD56 on NK cells may also be involved in cell adhesion, analogous to the function of N-CAM on neural cells. In the present report, we show that CD56 on human NK cells does function as an adhesion molecule in NK-target cell interaction.

Materials and Methods

mAbs. Unlabeled anti-Leu-19 mAb (4) was purchased from Becton Dickinson & Co. (Mountain View, CA). Unlabeled and phycoerythrin (PE)-conjugated NKH-1 mAbs (1) were purchased from Coulter Immunology (Hialeah, FL). Unlabeled anti-LFA-1 mAb (25.3) (7) was purchased from Cosmo Bio (Tokyo, Japan). Ascites containing anti-ICAM-1 mAb (RR1/1) (8) and those containing anti-LFA-3 mAb (TS2/9) (9) were kindly provided by Dr. T. A. Springer (Harvard Medical School, Boston, MA) and Dr. S. J. Burakoff (Dana-Farber Cancer Institute, Boston, MA), respectively.

Cells. The glioblastoma (U251MG, A172), neuroblastoma (GOTO), melanoma (G361), and leukemia (Molt-4, K562) cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). PBMC were isolated from the blood of healthy donors using Ficoll-Hypaque. After depleting monocytes and B cells by plastic adherence and passage through nylon wool, nonadherent lymphocytes were fractionated on discontinuous Percoll gradients as described (10). The low-buoyant density lymphocytes isolated from the interface between 30 and 35% consisted of 80–85% NKH-1$^+$ cells as estimated by flow cytometry and were served as the NK cells. NK cells were cultured for 18 h with 100 U/ml of rIL-2, as described (10), and then subjected to the cytotoxicity and conjugate formation assays.

Immunofluorescence and Flow Cytometry. Immunofluorescence and flow cytometric analysis

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were performed as described (10). In indirect immunofluorescence, FITC-conjugated goat anti-mouse Ig (F-GAM) antibody (Olympus, Tokyo, Japan) was used as the second antibody.

Cytotoxicity Assay. Cytotoxicity was measured using a standard 4 h ^{51}Cr release assay as described (10).

Conjugate Formation Assay. Untreated or mAb-treated NK cells (10^6) and target cells (2×10^5) were mixed in a final volume of 100 μl and centrifuged at 500 *g* for 1 min. After a 20-min incubation at room temperature, the cells were gently resuspended and counted under microscopy. NK cells and target cells were clearly discriminated by their distinctive difference in cell size. Target cells binding at least two NK cells were scored as positive and 200 target cells were counted in triplicate.

Results and Discussion

Recently, two antigen-independent pathways of effector-target cell binding have been demonstrated that are mediated by the interaction between CD2 on effector cells and LFA-3(CD58) on target cells, and by that between LFA-1 (CD11a/CD18) on effector cells and ICAM-1 (CD54) on target cells (11). Relative contribution of either pathway to cell binding is variable among combinations of effector and target cells (11). These two adhesion pathways are involved also in NK cell interaction with target cells (12). To assess the putative adhesive function of CD56 on NK cells in a correlation with these known two pathways, we first examined the expression of CD56 among various target cells in parallel with those of LFA-3 and ICAM-1. Several tumor cell lines of neuroectodermal origin (U251MG and A172 glioblastomas, GOTO neuroblastoma, and G361 melanoma) were revealed to express CD56 as estimated by flow cytometry with NKH-1 mAb, although Molt-4 and K562 leukemia cell lines were $CD56^-$ (data not shown). This preferential expression of CD56 on neural tumor cell lines is consistent with the recent finding that CD56 is identical to N-CAM (5). Among them, we used a glioblastoma cell line U251MG ($NKH\text{-}1^+$, $ICAM\text{-}1^-$, $LFA\text{-}3^+$) (Fig. 1) and a T acute lymphoblastic leukemia (T-ALL) cell line Molt-4 ($NKH\text{-}1^-$, $ICAM\text{-}1^+$, $LFA\text{-}3^+$) (Fig. 1) in the following studies, since they were highly susceptible for activated NK cells prepared by culturing low-buoyant density PBL with rIL-2 for 18 h.

We then examined the inhibitory effect of an anti-CD56 mAb (anti-Leu-19) on NK cell-mediated cytotoxicity against these two targets in conjunction with an anti-LFA-3 mAb (TS2/9) and/or an anti-LFA-1 mAb (25.3). Anti-LFA-3 mAb was preferred rather than anti-CD2 mAb to abrogate the CD2/LFA-3 interaction since anti-CD2 mAb may activate NK cell cytotoxicity in some circumstances (12). Anti-LFA-1 mAb was also preferred rather than anti-ICAM-1 mAb since ICAM-1 is not the sole ligand for LFA-1 (13). As shown in Fig. 2, NK cell-mediated cytolysis against the $CD56^+$ target U251MG was only partially inhibited by anti-LFA-1 or anti-LFA-3 mAb alone and also by the combination of both anti-LFA-1 and anti-LFA-3 mAbs. In contrast, the cytotoxicity against the $CD56^-$ target Molt-4 was also partially blocked by anti-LFA-1 or anti-LFA-3 mAb alone, however, was completely abrogated by the combination of both anti-LFA-1 and anti-LFA-3 mAbs. Anti-Leu-19 mAb, when used alone, did not inhibit the NK cytotoxicity against U251MG and Molt-4. However, when used in combinations with anti-LFA-1 and/or anti-LFA-3 mAbs, anti-Leu-19 mAb did exhibit synergistic inhibitory effects, and a complete inhibition was attained only after the treatment with all the three mAbs, when $CD56^+$ U251MG was used as the target (Fig. 2). No such synergistic inhibitory effect of anti-Leu-19 mAb was

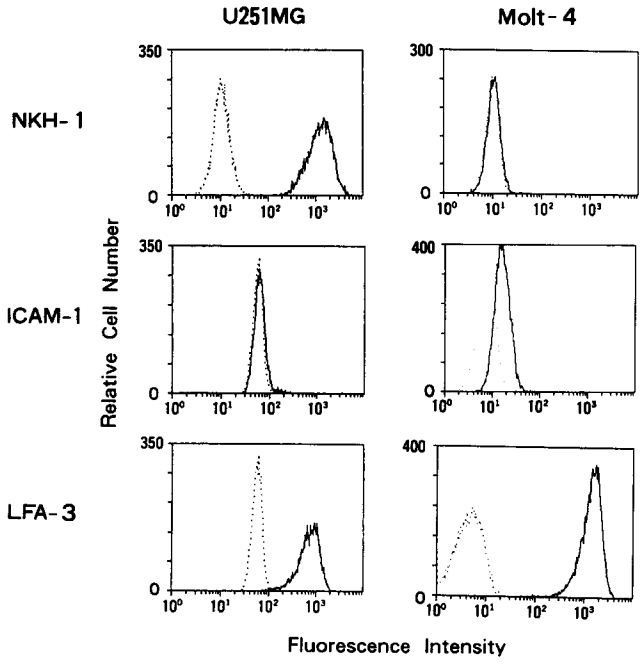

FIGURE 1. Expression of NKH-1, ICAM-1, and LFA-3 on U251-MG and Molt-4 target cells. A glioblastoma cell line (U251MG) and a T-ALL cell line (Molt-4) were reacted with anti-NKH-1 mAb (N901; 1 μg), anti-ICAM-1 mAb (RR1/1; 1:200 dilution of ascites), or anti-LFA-3 mAb (TS2/9; 1:500 dilution of ascites) followed by F-GAM. Samples were analyzed on a FACScan. Fluorescence profiles were generated from 10,000 cells (*solid lines*). Dotted lines indicate background staining with control IgG1 mAb.

observed when the CD56⁻ Molt-4 was used as the target (Fig. 2). A similar synergistic inhibitory effect with anti-LFA-1 and/or anti-LFA-3 mAbs was also observed when another anti-CD56 mAb, NKH-1, was used and when CD56⁺ GOTO, but not CD56⁻ K562, were used as the targets (data not shown). These results indicate that CD56 on NK cells is involved in NK cell-mediated cytotoxicity when the target cells express CD56.

We next examined the effect of anti-Leu-19 mAb on NK cell binding to CD56⁺ or CD56⁻ target cells in conjunction with anti-LFA-1 and/or anti-LFA-3 mAbs. As shown in Fig. 3, synergistic inhibitory effects of anti-Leu-19 mAb with anti-LFA-1 and/or anti-LFA-3 mAbs, similar to those observed in NK cell-mediated cytotoxicity (Fig. 2), were observed again on NK cell binding to target cells only when

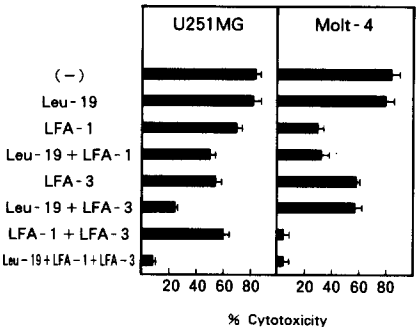

FIGURE 2. Inhibitory effect of anti-Leu-19, anti-LFA-1, and/or anti-LFA-3 mAbs on NK cell-mediated cytotoxicity. Low-buoyant density PBL highly enriched for NK cells were cultured with 100 U/ml of rIL-2. After an 18-h culture, effector NK cells were preincubated with or without anti-Leu-19 mAb (10 μg/ml) and/or anti-LFA-1 mAb (25.3; 10 μg/ml) for 30 min at 37°C, and then cytotoxicity was tested against U251MG or Molt-4 target cells, which were preincubated with or without anti-LFA-3 mAb (TS2/9; 1:100 dilution of ascites) for 30 min at 37°C, at an E/T ratio of 5:1.

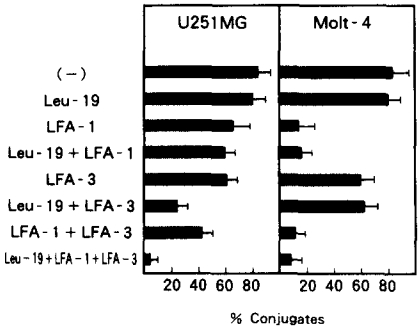

FIGURE 3. Inhibitory effect of anti-Leu-19, anti-LFA-1, and/or anti-LFA-3 mAbs on conjugate formation between NK cells and target cells. NK cells and U251MG or Molt-4 target cells preincubated with anti-Leu-19, anti-LFA-1, and/or anti-LFA-3 mAbs, as described in Fig. 2, were mixed at an E/T ratio of 5:1, and centrifuged. After a 20-min incubation at room temperature, the cells were gently resuspended and counted. Results are indicated as mean percent target cells binding at least two NK cells after counting 200 target cells in triplicate.

$CD56^+$ U251MG was used as the target. A similar inhibitory effect of anti-Leu-19 mAb on NK cell binding to target cells was also observed when either the effector NK cells or the target U251MG cells was pretreated with anti-Leu-19 mAb, washed, and then mixed together (data not shown), demonstrating that anti-Leu-19 mAb blocks NK cell binding at both the effector and the target sites. These results indicate that CD56 on NK cells does contribute to NK cell binding when the target cells express CD56, and suggest that the binding is homotypic, i.e., CD56 on NK cells binds to CD56 on target cells. This is consistent with the homotypic binding of N-CAM demonstrated in neural and muscle tissues.

From these results, it is evident that CD56 on NK cells is involved in NK-target cell interactions. The contribution of CD56 to NK cell cytotoxicity and binding could be demonstrated only when the target cells express CD56. This explains the failure to demonstrate an inhibitory effect of anti-Leu-19 and NKH-1 mAbs on NK cytotoxicity in the previous studies (1–4), in which only $CD56^-$ cells, such as K562 and Molt-4, were used as the targets. In addition, the inhibitory effects of anti-Leu-19 mAb could be demonstrated only in the presence of anti-LFA-1 and/or anti-LFA-3 mAbs. This indicates that the homotypic interaction between CD56 (N-CAM) on NK and target cells constitutes the third pathway of NK-target cell binding, other than the two adhesion pathways mediated by the LFA-1/ICAM-1 and CD2/LFA-3 interactions, although the NK cell binding to $CD56^-$ target cells is predominantly mediated by the latter two pathways.

It is evident from our data that CD56 on NK cells can function as an adhesion molecule, however, the physiological significance of the rather confined expression of N-CAM to NK cells and a minor subset of T lymphocytes mediating MHC-unrestricted cytotoxicity in peripheral blood remains to be determined. Since N-CAM is expressed in various tissues (6), the adhesive function of CD56 on the particular lymphocytes may be directly involved in their interaction with these tissues and cells expressing N-CAM. Further studies will be needed to test this possibility.

Summary

The CD56 differentiation antigen, recognized by anti-Leu-19 and NKH-1 mAbs, is a 200–220-kD glycoprotein that is expressed predominantly on human NK cells and a minor subset of T lymphocytes mediating MHC-unrestricted cytotoxicity. The recent finding that CD56 is an isoform of the neural cell adhesion molecule

(N-CAM) prompted us to examine the adhesive function of CD56 in the NK–target cell interaction. Synergistic inhibitory effects of anti-CD56 mAbs with anti-LFA-1 and/or anti-LFA-3 mAbs were demonstrated on NK cell–mediated cytotoxicity and on NK cell binding to target cells only when the target cells also express CD56. These findings indicate that CD56 on NK cells can serve as the third pathway of cell adhesion other than those mediated by the CD2/LFA-3 and LFA-1/ICAM-1 interactions, and is involved in NK cell cytotoxicity when interacting with the cells bearing N-CAM.

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References

1. Griffin, J. D., T. Hercend, R. Beveridge, and S. F. Schlossman. 1983. Characterization of an antigen expressed by human natural killer cells. *J. Immunol.* 130:2947.
2. Hercend, T., J. D. Griffin, A. Bensussan, R. E. Schmidt, M. A. Edson, A. Brennan, C. Murray, J. F. Daley, S. F. Schlossman, and J. Ritz. 1985. Generation of monoclonal antibodies to human natural killer clone: characterization of two natural killer associated antigens, NKH1a and NKH2, expressed on subsets of large granular lymphocytes. *J. Clin. Invest.* 75:932.
3. Schmidt, R. E., C. Murray, J. F. Daley, S. F. Schlossman, and J. Ritz. 1986. A subset of natural killer cells in peripheral blood display a mature T cell phenotype. *J. Exp. Med.* 164:351.
4. Lanier, L. L., A. M. Le, C. I. Civin, M. R. Loken, and J. H. Phillips. 1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J. Immunol.* 136:4480.
5. Lanier, L. L., R. Testi, J. Binal, and J. H. Phillips. 1989. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *J. Exp. Med.* 169:2233.
6. Edelman, G. M. 1986. Cell adhesion molecules in the regulation of animal form and tissue pattern. *Annu. Rev. Cell Biol.* 2:81.
7. Mazerolles, F., C. Lumbroso, O. Lecomte, F. L. Deist, and A. Fischer. 1988. The role of lymphocyte function associated antigen 1 (LFA-1) in the adherence of T lymphocytes to B lymphocytes. *Eur. J. Immunol.* 18:1229.
8. Rothlein, R., M. L. Dustin, S. D. Marlin, and T. A. Springer. 1986. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *J. Immunol.* 137:1270.
9. Sanchez-Madrid, F., A. M. Krensky, C. F. Ware, E. Robbins, J. L. Strominger, S. J. Burakoff, and T. A. Springer. 1982. Three distinct antigens associated with human T lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3. *Proc. Natl. Acad. Sci. USA.* 79:7489.
10. Yagita, H., M. Nakata, A. Azuma, T. Nitta, T. Takeshita, K. Sugamura, and K. Okumura. 1989. Activation of peripheral blood T cells via the p75 interleukin 2 receptor. *J. Exp. Med.* 170:1445.
11. Shaw, S., and G. E. G. Luce. 1987. The lymphocyte function-associated antigen (LFA)-1 and CD2/LFA-3 pathways of antigen-independent human T cell adhesion. *J. Immunol.* 139:1037.
12. Ritz, J., R. E. Schmidt, J. Michon, T. Hercend, and S. F. Schlossman. 1988. Characterization of functional surface structures on human natural killer cells. *Adv. Immunol.* 42:181.
13. Staunton, D. E., M. L. Dustin, and T. A. Springer. 1989. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. *Nature (Lond.).* 339:61.