FULL PAPER *Immunology*

CD56 Expression in Feline Lymphoid Cells

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ABSTRACT. The neural-cell adhesion molecule (N-CAM) consists of three major types of polypeptides (180, 140, and 120 kDa) whose predominant differences exist within the transmembrane and cytoplasmic domains. In this study, we generated a monoclonal antibody, termed SZK1, reactive to feline CD56 (fCD56) molecules (140 kDa form of N-CAM) expressed by the baculovirus expression system and investigated fCD56 expression in feline lymphoid cells. In flow cytometric analysis, SZK1 was reactive to a feline T-lymphoblastoid cell line MYA-1. Further, SZK1 was reactive to a very small population (1.1–1.7%) of freshly isolated peripheral blood lymphocytes (PBLs) of three specific pathogen-free cats, and the reactivity was increased by culturing of PBLs in the presence of interleukin-2 following concanavalin A-stimulation (>10%). In immunoblotting analysis, SZK1 detected an approximately 160 kDa antigen from MYA-1 cells, while from RNA of the cells reverse transcription-polymerase chain reaction amplified the fragment resembling 140 kDa form of N-CAM. These finding suggest that fCD56 has similar characteristics with human CD56.
KEY WORDS: CD56, feline, lymphoid cell.

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The neural-cell adhesion molecule (N-CAM) is a member of the immunoglobulin superfamily that mediates homotypic adhesive interactions of cells in neural and muscle tissues [3]. N-CAM consists of three major types of polypeptides (180, 140, and 120 kDa, estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)) generated by alternative mRNA splicing from a single gene in mammalians and avians [1, 6, 15, 16, 25]. The predominant differences in these forms are within the transmembrane and cytoplasmic domains [3]. The 140 kDa form lacks an insert of approximately 270 amino acids in the cytoplasmic region, which is present within the 180 kDa form [4]. The 120 kDa form lacks a membrane spanning domain and is linked to a glyco-phosphatidylinositol [7]. In addition, the three forms have the optional sequences within extracellular domains that arise by the splicing of smaller RNA segments and correspond to 10–40 amino acids [4]. The form(s) of N-CAM that is expressed seems to be dependent on the developmental-stages and cell types [4].

In humans, CD56 (Leu19, NKH-1) antigen is expressed on approximately 15% of peripheral blood lymphocytes (PBLs) that are mainly composed of natural killer (NK) cells (–10%) and $CD3^+$ T lymphocytes (<5%) [10]. Both $CD56^+$ NK cells and $CD56^+$ T cells mediate non-major histocompatibility complex (MHC)-restricted cytotoxicity [8, 10, 26]. CD56 has been shown to be identical to the 140 kDa form of N-CAM [12], however the antigen as well as other forms of N-CAM undergoes posttranslational modifications including addition of polysialic acids in some cell-types such as lymphocytes, resulting in showing larger molecular weight [4, 8, 10, 12].

Little is known about feline NK cells or CD56 expression in feline T cells. Recently we cloned a cDNA encoding feline homologue of N-CAM from a feline thymus cDNA library [17], that corresponds to 140 kDa form at the level of the gene. In the present study, we generated a monoclonal antibody (mAb) to fCD56, termed SZK1, and examined the expression and molecular size of the antigen recognized by SZK1 mAb in feline lymphoid cells by flow cytometry (FCM), immunoblotting, and reverse transcription-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Cell culture: MYA-1 cells, an interleukin-2 (IL-2) dependent feline T-lymphoblastoid cell line [14], were cultured in RPMI 1640 growth medium supplemented with heat-inactivated foetal calf serum (FCS, 10%), 2-mercaptoethanol (50 μM), polybrene (2 μg/m*l*), recombinant human IL-2 (100 units/m*l*) and antibiotics at 37°C in a humidified atmosphere of 5% CO_2 in air. The culture was passaged every three or four days to achieve approximately 5×10^5 cells/m*l*. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-paque™ (Amersham Pharmacia Biotech, Uppsala, Sweden) from heparin-treated whole blood of three 11 to 13-years old, specific pathogen-free (SPF) cats (Cats 201, 102 and 202 obtained from Dr. K. Nakano, Kitasato University). PBMCs were stimulated by concanavalin A (Con A, 10 μg/m*l*) for three days and maintained as described for

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MYA-1 cells.

mAb: mAbs to fCD56 were generated as described previously [22]. Briefly, BALB/c mice were immunized with an insect cell line, High Five™ cells (Invitrogen, NV Leek, the Netherlands), infected with the recombinant baculovirus rAcfCD56F140 [18], which contains *fCD56* cDNA (140 kDa form of N-CAM) under the control of a polyhedrin promoter. Hybridomas, which were generated by the fusion of mouse spleen cells with P3X63Ag8U.1 myeloma, were screened with Con A-stimulated, cultured feline PBLs by indirect fluorescence assay (IFA) [22]. The isotype of antibody was determined by IsoDetect™ mouse mAb isotyping kit (Stratagene, La Jolla, CA).

Immunoblotting: Cells were lysed with sample buffer (2% SDS, 0.0625 M Tris-HCl pH6.8, 20% glycerol, and 5% 2-mercaptoethanol) and boiled for 3 min. The lysates were subjected to SDS-PAGE (7.5% gel) and transferred onto Hybond-C extra (Amersham Pharmacia Biotech). The membrane was incubated with antibodies (1:2000 dilution of mouse ascitic fluids), and the antibodies on the membrane were detected using goat anti-mouse IgG + IgM conjugated with horse radish peroxidase (Biosource, Camarillo, CA) together with 3, 3'-diaminobenzidine, tetra hydrochloride or enhanced chemiluminescence (Amersham Pharmacia Biotech).

FCM: FCM analysis was performed as described previously [23, 24]. Cells were washed with wash buffer (phosphate-buffered saline containing 2% FCS and 0.1% NaN_3) and incubated with mAbs. After being washed with the buffer, the cells were incubated with a fluorescein-conjugated goat IgG fraction to mouse immunoglobulins (IgG, IgA, IgM) (Cappel, Aurora, OH). After further washing, the cells were analyzed with a FACScan flow cytometer and Cell Quest software (Becton Dickinson, San Jose, CA). For two color FCM analysis, anti-fCD56 mAb (SZK1, IgG1) was labeled with fluorescein-isothiocyanate (Sigma, St. Louis, MO) and then used in combination with unlabeled anti-fCD4 (45B4, IgG2a) [22] or anti-fCD8α (2D7, IgG2a) [23]. The unlabeled antibodies were detected by rat anti-mouse IgG2a conjugated with R-Phycoerythrin (Zymed Laboratories, San francisco, CA). It was confirmed that the IgG2a-specific antibody does not cross-react with SZK1 (IgG1) or with other IgG1 mAbs (data not shown).

Panning: A polystyrene Petri dish (Bio-Bik, Osaka, Japan) was incubated at 4°C overnight with a goat anti-mouse IgG (Rockland, Gilbertsville, PA) prepared at 10 μg/m*l* in phosphate-buffered saline (PBS). After being washed with PBS containing 2% FCS (2FCS-PBS), the dish was further incubated at 37°C for 30 min with 10 μ*l* ascite of SZK1 mAb in 4 m*l* of 2FCS-PBS. The dish was washed with 2FCS-PBS and then used for panning. MYA-1 cells (5×10^6 in 4 m*l* of culture medium) were seeded on the mAb-coated Petri dish and incubated at 4°C for 90 min. Non-adherent cells were harvested as $fCD56^-$ MYA-1. After the dish was washed well with 2FCS-PBS, adherent cells were harvested as $fCD56^+$ MYA-1 by cell scrapers.

RT-PCR: Total RNA was prepared from MYA-1 cells by ISOGEN-LS (Nippon Gene, Tokyo, Japan) and cDNA was synthesized using an oligo (dT) primer and Super Script II Reverse Transcriptase (Gibco BRL, Gaithersburg, MD). For the subsequent PCR, we used the primers fCD56/3'-2 5'-CAGGCACTACCTGGTCAAGTACCGAG-3' (nucleotide (nt) position 2102–2127) and fCD56R2 5'-TCTGCTCATTTCTTGTCACCCATCA-3' (nt position 2775–2751). Both primers have been used for the cloning of *fCD56* cDNA [17]. Amplified fragments obtained with the primer pair from the 140 kDa N-CAM would be approximately 650 bp. In the case of the 180 kDa form, the amplified fragments would be more than 1.4 kbp long due to an additional exon (approximately 800 bp [4]).

RESULTS

mAb to fCD56: From hybridomas obtained from mice immunized with rAcfCD56F140-infected insect cells, one clone SZK1 (IgG1) was established according to its reactivity with cultured feline PBLs. The mAb was reactive to rAcfCD56F140-infected cells, but not to control baculovirus-infected cells in IFA (data not shown). In the immunoblotting analysis, SZK1 mAb detected a broad band ranging from 130 to 140 kDa in the rAcfCD56F140-infected cells, but not in the control baculovirus-infected Sf9 cells (Fig. 1).

CD56 expression in feline lymphoid cells: No apparent $fCD56^+$ population was found in feline PBLs freshly isolated from three SPF cats by FCM with SZK1 (data not shown). However, a feline T-lymphoblastoid MYA-1 cell line, which has been established by long term (over 11 months) culture of feline PBLs with IL-2 after Con A-stimulation [14], showed approximately 20% reactivity to fCD56 mAb (Fig. 2A). To determine whether the culture increases fCD56 expression, feline PBLs were stimulated

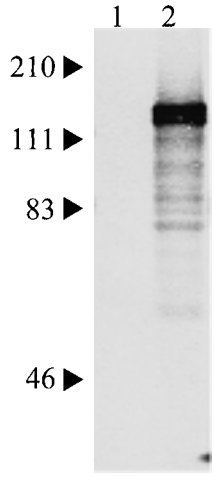

Fig. 1. Reactivity of anti-fCD56 mAb SZK1 to control baculovirus (lane 1)- or rAcfCD56F140 (lane 2)-infected Sf9 cells on immunoblotting. Numbers on the left represent molecular size markers (kDa). The results are very similar to those for anti-human CD56 mAb (YLEM, Roma, Italy) [18].

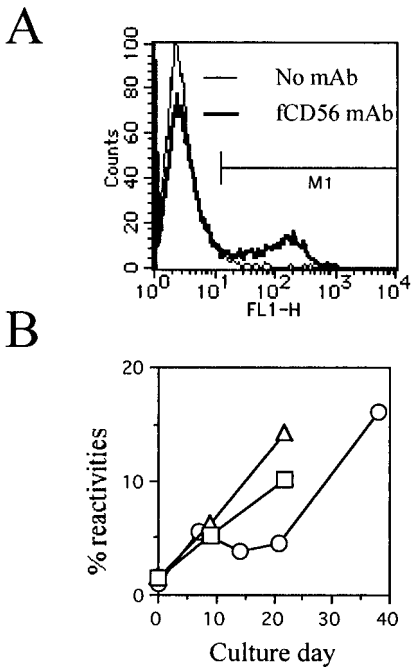

Fig. 2. fCD56 expression in a feline T-lymphoblastoid cell line MYA-1 (A) and cultured feline PBLs (B) in FCM. Feline PBLs were stimulated by Con A and cultured in the presence of IL-2, then fCD56 expression was examined on the days indicated (B). Three SPF cats were used: Cat 201 (○), Cat 102 (△) and

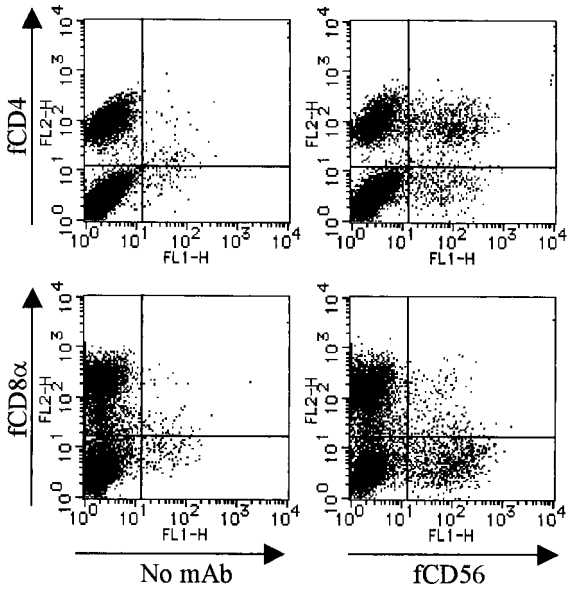

Fig. 3. fCD56 expression in $CD4^+$ and $CD8\alpha^+$ PBLs. Two color FCM was performed at day 22 (Cat 202).

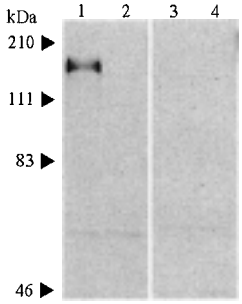

Fig. 4. Immunoblotting of fCD56 in MYA-1 cells. MYA-1 cells were separated into two types, $fCD56^+$ (lanes 1 and 3) and $fCD56^-$ (lanes 2 and 4), by a panning method and used with anti-fCD56 (lanes 1 and 2) or irrelevant (lanes 3 and 4) mAbs. Numbers on the left represent molecular size markers.

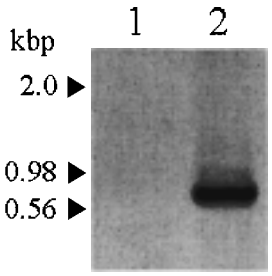

Fig. 5. RT-PCR to amplify a partial fragment of *fCD56* cDNA from total RNA of MYA-1 cells without (lane 1) or with (lane 2) RT. Numbers on the left represent molecular size markers.

and cultured as described for MYA-1 cells in the presence of IL-2, and then analyzed. As shown in Fig. 2B, the cultured feline PBLs showed 5.6% reactivity to fCD56 mAb as early as at day 7 of culture, and the percentage of $fCD56^+$ cells increased up to 16% at day 38, while fresh PBLs showed only 1.1–1.7 reactivities (day 0). In the indirect single color FCM analysis, no binding of normal mouse-serum to MYA-1 cells, fresh PBMCs or cultured PBMCs (days 7 and 38) were observed (data not shown) when compared with the cells treated only with secondary antibodies. In two color FCM at day 22, fCD56 expression was observed in both $fCD4^+$ and $fCD8\alpha^+$ cells (6–20% in $fCD4^+$ cells and 2–14% in $fCD8\alpha^+$ cells, Fig. 3).

Molecular size of fCD56: Immunoblotting analysis was performed for two types of cells, $fCD56^+$ (>98% $fCD56^+$ in FCM) and $fCD56^-$ (<5% $fCD56^+$) MYA-1 cells. SZK1 detected approximately 160 kDa molecules from $fCD56^+$ but not from $fCD56^-$ MYA-1 cells (Fig. 4). Control mAb did not detect any specific bands (Fig. 4). In RT-PCR, an approximately 650 fragment was amplified from MYA-1 RNA (Fig. 5).

DISCUSSION

In humans, unprimed, normal individuals have $CD56^+$ cells in periphery that comprise 10–15% of PBLs and exhibit non-MHC-restricted cytotoxicity [8, 10]. We could not detect apparent $CD56^+$ cell population in freshly isolated PBLs of SPF cats in this study (< 2%). Tompkins *et al.* [27] reported that PBMCs of clinical normal, nonimmune cats (4 to 9 years old) which had been in an isolation ward for over 3 years showed natural cytotoxic cell activities against virus-infected cells (*e.g.* 35% cytotoxicity at E:T ratio of 50:1). Hanlon *et al.* [5] reported that PBMCs of SPF cats (6 months old) showed NK-mediated cytotoxicities against hamster BHK cells (*e.g.* 10% cytotoxicity at E:T ratios of 50:1). CD56 expression is not essential to the cytotoxic function of human NK cells [9, 19], and further, to our knowledge, CD56 expression on murine and rat lymphoid cells including NK cells has not been reported. Therefore, it is unlikely that only a small population (< 2%), but not other population, exhibits such NK activities, and cats may have a $CD56^-$ population as MHC-unrestricted cytotoxicity-exhibiting cells in addition to $CD56^+$ population. Identification of distinct population(s) exhibiting MHC-unrestricted activity might be possible by using other cell antigens such as NK markers [21] and CD57 [28].

In addition to the MYA-1 cell line, both cultured $fCD4^+$ and $fCD8\alpha^+$ PBLs that have been stimulated by Con A and cultured in the presence of IL-2, expressed fCD56 antigens. MYA-1 cells express CD3ε (>98% in indirect FCM, data not shown) and are IL-2-dependent [14]. The cultured PBLs had also the same characteristics (data not shown). The significance of fCD56 expression in MYA-1 cells or the cultured PBLs is yet to be clarified, however the finding is consistent with Lanier's report that CD56 is expressed on most $CD4^+$ and $CD8^+$ IL-2-dependent human T cell lines and clones that have been maintained in long term culture [11]. However, it is unclear whether $fCD56^+$ cells in cultured PBLs are the lineage of fresh $fCD56^+$ PBLs or not. Further experiments using purified $fCD56^+$ PBLs or fCD56-depleted PBLs will be needed.

The molecular size of fCD56 expressed in MYA-1 cells was larger than 140 kDa (Fig. 4), therefore, we examined the cytoplasmic region of N-CAM expressed in MYA-1 cells by RT-PCR. While there are small variants (approximately 40 amino acids at most) within the extracellular domain, the changes of molecular weight affected by them are slight compared with those occurred within cytoplasmic region (270 amino acids) in other animals. We detected a 650 bp fragment from the cDNA of MYA-1 cells, indicating that the amplified fragment corresponds to a partial sequence of the 140 kDa form which does not contain an insertion (exon 18 in other animals [1, 6, 15, 20]) specific for the 180 kDa form within the fragment. Thus, as in humans, fCD56 molecules of MYA-1 cells might be structurally identical to the 140 kDa form of N-CAM but have a greater molecular weight (160 kDa in appearance) because of a modification with, for example, abundant sialic acid residues [12]. This speculation is also supported by the result in the insect expression system in which proteins are not usually sialylated [13]; as shown in Fig. 1, "140 kDa form"-coding baculovirus produces approximately 140 kDa molecules in insect cells detected by the anti-fCD56 mAb. The difference of molecular weight between MYA-1 and insect cells was also observed when analyzed using the same gel for immunoblotting (data not shown).

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