Feline Hybridoma Growth Factor/Interleukin-6 Activity

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An assay system was developed to measure feline hybridoma growth factor (HGF)/interleukin-6 (IL-6) activity in biological samples containing many kinds of cytokines by using the proliferation of the newly established mouse-rat hybridoma clone, B3B1. The proliferative response of this B3B1 clone was IL-6-specific, and could not be promoted by other cytokines including IL-1, IL-2, IL-3, and granulocyte-colony-stimulating factor (G-CSF). The anti-human B-cell stimulatory factor 2 (BSF-2)/IL-6 antiserum did not neutralize feline HGF/IL-6 activity in conditioned media prepared from feline con A-stimulated splenocytes and unstimulated alveolar macrophages, indicating antigenic differences between species. Feline HGF/IL-6 was eluted into the fractions corresponding to a molecular weight of 30,000-40,000 in gel filtration, and into the fractions at a salt concentration of 0.2-0.3 M NaCl in anion exchange chromatography. The physicochemical properties of feline HGF/IL-6 were slightly different from those of murine and human IL-6.

Key words: B cell, cat, cytokine

INTRODUCTION

Cats are susceptible to infection with oncogenic retrovirus [18] and T-lymphotropic lentivirus [9], which lead to an immunodeficiency syndrome very similar to human disorders. Despite the usefulness of cats as a model for preclinical studies of human diseases, little is known about feline immunoregulatory systems. We have previously characterized several feline cytokines including interleukin 1 (IL-1) [6] and IL-2 [4], and reported that the production of these cytokines was altered in feline leukemia virus (FeLV) infection [5] and coronavirus-induced feline infectious peritonitis (FIP) [7]. Some reports have suggested that B-cell abnormalities were associated with FeLV infection [15, 18] and FIP [8]. However, precise studies on the aspect of cytokines that regulates B-cell growth and differentiation have not yet been done.

Out of several B-cell tropic cytokines, B-cell stimulatory factor-2 (BSF-2)/interleukin 6 (IL-6) has been extensively investigated recently, because of its multiple bioactivities, such as the induction of terminal differentiation of B cells into antibody-secreting cells [10, 11], growth factor for hybridomas [20], and plasmacytomas, and the induction of acute-phase protein synthesis by the liver [3].

In the present study, therefore, a factor-dependent hybridoma cell clone that responds to feline IL-6 was established to obtain basic information on feline IL-6.

MATERIALS AND METHODS

Cytokines

Human recombinant IL-1β was obtained from the Genzyme Corporation (Boston, MA). Human recombinant IL-2 was kindly provided by Shionogi Pharmaceutical Corporation (Tokyo, Japan). Human recombinant granulocyte-colony-stimulating factor (G-CSF) was kindly provided by Kirin Brewery Co. Ltd. (Maebashi, Japan). Human recombinant (BSF-2)/IL-6 [1] was kindly provided by Dr. M. Mitani (Biotechnology Research Laboratory, Tosoh Co., Kanagawa, Japan). Murine IL-3 was partially purified from culture supernatants of WEHI-3 cells (kindly donated by Dr. S. Kyuwa, Institute of Medical Science, University of Tokyo) by DEAE-cellulose chromatography as previously described [12].

Preparation of Feline and Murine Conditioned Media (CM)

Conditioned media (CM) from concanavalin A (con A; Sigma Chemical Co., St. Louis, MO) stimulated feline and murine splenocytes were prepared according to the method described previously [17]. Briefly, 2 × 10⁷ cells/ml of splenocyte suspensions were cultured for 2 hr

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Fig. 1. B3B1 cell proliferation-promoting activity in feline CM (a) and murine CM (b) prepared from con A-stimulated splenocytes and alveolar macrophages. B3B1 cells were cultured in the presence of fivefold diluted CM prepared from con A-stimulated splenocytes (●) or alveolar macrophages (▲). Cultures of B3B1 cells with medium alone (□) were examined for background response. Cell numbers were evaluated by hexosaminidase levels on day 4 in triplicate cultures.

Fig. 2. Effect of murine and human cytokines on proliferative response of B3B1 cells. B3B1 cells were cultured in the presence of fivefold diluted various cytokines including human recombinant IL-1 (10 U/ml; ●), IL-2 (50 U/ml; ▲), IL-6 (100 U/ml; ◊), G-CSF (1.25 μg/ml; □), and murine purified IL-3 (△). Cultures of B3B1 cells with medium alone (□) were examined for background response. Cell numbers were evaluated by hexosaminidase levels on day 4 in triplicate cultures.

cells/ml in RPMI 1640 medium with 10% FBS. After a 90 min incubation in plastic Petri dishes, nonadherent cell fractions were removed by repeated washing with PBS. The relative proportion of residual adherent cell fractions was greater than 95% macrophages, as determined by nonspecific esterase staining. These cultures were incubated for 24 hr, and then the supernatants were collected. The resulting supernatants were stored at −20°C until used.

Preparation of Hybridoma Clone

HGF-dependent hybridoma cell clones were prepared according to the method described earlier [20], with a minor modification. Briefly, splenocytes (1 × 10⁸ cells) from a Lewis rat injected intraperitoneally with lipopolysaccharide (LPS; E. coli serotype 0.55:B5, Difco Laboratories, Detroit, MI) were fused with azaguanine-resistant X-63 myeloma cells (1 × 10⁵ cells) in the presence of polyethylene glycol 6000 (Sigma). Fused cells were cultured in RPMI 1640 medium with 10% FBS, 100 μM hypoxanthine, 0.4 μM aminopterin, 10 μM thymidine, and 20 μg/ml gentamicin. For the preparation of HGF-dependent clones, an aliquot of the CM prepared from con A-stimulated feline splenocytes was further added to the medium described above.

Hybridoma Cell Proliferation Assay

To determine the HGF sensitivity of freshly prepared hybridoma cells, these hybridoma cells (3,000 cells/well) were cultured for 3 days at 37°C in each well of a 96-well flat-bottomed microtiter plate (Falcon 3072; Becton Dickinson Labware, Oxnard, CA) in the presence or the absence of the culture supernatants of con
Fig. 3. Effect of anti-human BSF-2/IL-6 antiserum on proliferative response of B3B1 cells to feline CM. B3B1 cells were cultured at various dilutions of rabbit anti-human BSF-2/IL-6 antiserum in the presence of recombinant human BSF-2/IL-6 (2 U/ml; ©) or feline CM prepared from con A-stimulated splenocytes (•) and alveolar macrophages (△) at a 1:10 dilution, respectively. Cell numbers were evaluated by hexosaminidase levels on day 4 in triplicate cultures.

Fig. 4. Gel filtration profiles of feline HGF/IL-6 in the CM on an FPLC-Superose 12 column. The tenfold concentrated CM (0.2 ml) prepared from con A-stimulated splenocytes (A) and alveolar macrophages (B) were applied to a Superose 12 column and eluted with PBS at a flow rate of 0.5 ml/min. Each fraction was tested for B3B1 cell proliferation-promoting activity (shaded area). The results of B3B1 cell proliferation represent arithmetic means of triplicate cultures, which were subtracted from the background B3B1 cell proliferation with medium alone. Bovine serum albumin (67,000), ovalbumin (43,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700) were used as MW markers.

A-stimulated feline splenocytes. The cells were pulsed with 0.4 μCi/well of tritiated thymidine (3H-TdR; New England Nuclear, Boston, MA) for the last 6 hr, and harvested onto glass-fiber filters (Labo Science Co., Japan). The amount of incorporated radioactivity was quantified by a liquid scintillation counter (Aloka-LSC 2000; Aloka Co., Japan).

For measuring the HGF activity, the hybridoma cell clone B3B1 was used as the indicator cell, unless otherwise stated. B3B1 cells (3,000 cells/well) were cultured
in the serially fivefold diluted samples in each well of 96-well flat-bottomed microtiter plates. After culture for 4 days at 37°C in a 5% CO₂-humidified atmosphere, the cell growth was determined by hexosaminidase assay, as described by Landegren [13].

**Inhibition Test**

To determine the specificity and cross-reactivity of B3B1 cell proliferation, a B3B1 cell proliferation assay was performed at various dilutions of rabbit anti-human BSF-2/IL-6 antiserum, which was a kind gift from Dr. M. Mitani (Biotechnology Research Laboratory) in the presence of recombinant-human IL-6 or feline CM.

**Gel Filtration**

The CM, concentrated tenfold by ultrafiltration (0.2 ml), were applied to a Superose 12 column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with PBS containing 10⁻⁴ (vol/vol) Tween 20 (Sigma) operated by a FPLC system (Pharmacia). The sample was eluted into 0.5 ml fractions with PBS at a flow rate of 0.5 ml/min. Aliquots of the fractions were sterilized by Millipore filters (0.22 μm; Millipore Co., Bedford, MA) and assayed for HGF activity.

The column was calibrated with the following molecular weight (MW) markers (Pharmacia); bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), chymotrypsinogen A (MW 25,000), and ribonuclease A (MW 13,700).

**Anion Exchange Chromatography**

Anion exchange chromatography was performed on a Mono Q column (Pharmacia) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 20 mM NaCl, using the FPLC system (Pharmacia). The tenfold concentrated CM (0.5 ml) were applied to the column and washed with starting buffer for 5 min, and the material bound to the column was eluted at 1.0 ml/min with a linear gradient of increasing salt concentration from 20 mM to 0.5 M NaCl in 20 mM Tris-HCl buffer (pH 8.0). All fractions were dialyzed extensively against PBS, sterilized by Millipore filters (0.22 μm), and then assayed for HGF activity.
RESULTS

Cloning of HGF-Dependent Hybridoma Cell Clone

After 14 days in culture, the number of wells showing hybridoma growth was 15 times higher in the plates containing 10% con A-stimulated feline splenocyte-derived CM than in the control plates without CM. Out of these hybridoma cells, seven hybridoma cell clones were screened for their growth activity to the CM. Three clones strongly proliferated in response to the CM, while they did not proliferate significantly in the absence of the CM. The proliferation of the other four clones was not affected by addition of the CM (data not shown). Thus, the former three hybridoma cells were selected for further cloning by limiting dilution. A panel of hybridoma clones was derived by limiting dilution in the presence of the 10% CM from con A-stimulated feline splenocytes. A strictly CM-dependent clone, B3B1, was used as an indicator cell for the following HGF assay.

Proliferative Response of B3B1 Cells to Feline and Murine CM

Growth profiles of B3B1 cells in response to the feline CM from a representative experiment are shown in Figure 1a. The dose-dependent proliferation was observed in response to the CM from con A-stimulated splenocytes with a maximal proliferation at a dilution of 1:2. B3B1 cells also proliferated to the CM from alveolar macrophages in a manner similar to the CM from con A-stimulated splenocytes.

Proliferative Response of B3B1 Cells to Recombinant and Purified Cytokines

To address the factor responsible for B3B1 cell growth in these feline and murine CM, the effects of recombinant human and purified murine cytokines on B3B1 cell proliferation were examined. As shown in Figure 2, any concentration of human IL-1, IL-2, G-CSF, and murine IL-3 failed to promote B3B1 cell proliferation. The dose-dependent proliferation of B3B1 cells was only observed when they were stimulated with human recombinant BSF-2/IL-6.

Inhibition Test of B3B1 Cell Proliferation by Anti-Human BSF-2/IL-6 Antiserum

By an inhibition test using rabbit anti-human BSF-2/IL-6 antiserum (Fig. 3), human recombinant BSF-2/IL-6-induced B3B1 cell proliferation was inhibited by anti-human BSF-2/IL-6 antiserum at dilutions from 1:2, 000 to 1:200, with complete inhibition occurring at a dilution of 1:400.

The effects of anti-human BSF-2/IL-6 antiserum on proliferative response of B3B1 cells induced by the feline CM were further examined. In contrast to human recombinant BSF-2/IL-6-induced proliferative response, the proliferation of B3B1 cells induced by the feline CM from both con A-stimulated splenocytes and alveolar macrophages was not suppressed by any dilution of anti-human BSF-2/IL-6 antiserum in the culture.

Physicochemical Properties of B3B1 Cell Proliferation-Promoting Factor in Feline CM

The elution profiles of the feline CM from con A-stimulated splenocytes and alveolar macrophages on a Superose 12 column are shown in Figure 4. A single peak of B3B1 cell proliferation-promoting activity was detected in the fractions corresponding to a MW ranging from 30,000 to 40,000 in both chromatographic profiles of the CM from con A-stimulated splenocytes and alveolar macrophages.

As shown in Figure 5, B3B1 cell proliferation-promoting activity was revealed in the fractions eluted from 0.2–0.3 M concentrations of NaCl in anion exchange chromatography. This elution profile of the CM from con A-stimulated splenocytes was approximately identical to that of the CM from alveolar macrophages.

DISCUSSION

In the present report, we have described HGF/IL-6 in feline CM prepared from both con A-stimulated splenocytes and nonstimulated alveolar macrophages, triggering the proliferation of a newly established factor-dependent murine hybridoma cell line, B3B1.

The proliferation of B3B1 cells was considered to be IL-6-specific, since B3B1 cells proliferated only in response to recombinant human BSF-2/IL-6 but not to other recombinant and purified cytokines such as IL-1, IL-2, IL-3, and G-CSF, and this BSF-2/IL-6-induced proliferation was inhibited specifically by the addition of anti-human BSF-2/IL-6 antiserum. Thus, the use of the B3B1 cell line gave us the tools to examine the nature of feline HGF/IL-6 in the crude CM-containing various immunologically active cytokines.

Until now, the only known growth factor for hybridomas was IL-6, although other workers have reported that IL-1, tumor necrosis factor (TNF) [14], and granulocyte-macrophage colony-stimulating factor (GM-CSF) [22] as well as IL-6 [21] were also growth factors for several clones of plasmacytomas. Since the bioactivity of GM-CSF was reported to be strictly species-specific [23], it is unlikely that the observed hybridoma cell growth-pro-
moting activity in feline CM was attributable to feline GM-CSF. Therefore, the findings in this study strongly suggest that the factor detected in the feline CM required for the proliferation of B3B1 cells was a feline counter-part of IL-6 in murine and human species.

We have, however, consistently observed that the proliferation of B3B1 cells in response to feline HGF/IL-6 could not be blocked by anti-human BSF-2/IL-6 antiserum. This finding suggests antigenic differences between human and feline HGF/IL-6 molecules with the same hybridoma growth-promoting activity.

Physicochemical analyses by gel filtration and anion exchange chromatography revealed that feline HGF/IL-6 could have biological activities similar to murine and human IL-6, although feline HGF/IL-6 was slightly distinct from murine and human IL-6 in molecular weight and anion exchange elution profile [11, 20]. Feline HGF/IL-6 was eluted into fractions corresponding to a MW of 30,000–40,000 in gel filtration, and into fractions at salt concentrations from 0.2 to 0.3 M NaCl, while murine and human IL-6 were reported to be eluted into fractions of MW 25,000–35,000 [2, 20] and salt concentrations from 0.1 to 0.2 M NaCl [20]. Whether these differences reside in the difference among species or the dimerization of protein is unclear. At this point, further purification of feline HGF/IL-6 is required for detailed characterization.

In a preliminary study, we already detected abnormally high levels of HGF/IL-6 activity in sera and ascites from cats with FIP showing hypergammaglobulinemia, suggesting a possible relation of feline HGF/IL-6 to polyclonal B-cell activation (manuscript in preparation).

IL-6 was found to be produced by various types of cells such as monocytes/macrophages [2], T cells [16], and fibroblasts [19], and abnormal production of IL-6 was thought to contribute to hypergammaglobulinemia and autoantibody production in certain human disorders [10]. Therefore, it is important to understand the mechanisms responsible for B-cell abnormalities and IL-6 production in feline disorders.

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