Feline Interleukin 2 Activity

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ABSTRACT. The interleukin 2 (IL-2) activity was found in the culture supernatants of feline lymphocytes stimulated with concanavalin A (Con A), as detected by the cell proliferation of a IL-2 dependent murine T cell line (CTLL-2). Feline IL-2 closely resembled rat and human IL-2 in the molecular weight of approximately 16000 daltons, as was estimated from Sephadex G-100 gel filtration chromatography studies. Feline IL-2 was labile to trypsin treatment and was rather sensitive to heating at 70°C for 15 min, the incubation at pH 3.2 and pH 10.5, and the treatment with urea (2, 4 and 8 M), as compared to those of human and murine IL-2. The maximal IL-2 activity in the supernatants from cultures in which lymphocytes were stimulated with Con A (10–100 µg/ml) appeared 6–12 hr after initiation of the culture. The addition of exogenous feline IL-2 resulted in the enhanced lymphocyte proliferation in response to Con A, whereas IL-2 alone did not have potent proliferative effects. The feline IL-2 activity was removed by a 4-hr incubation with lymphocytes stimulated with Con A for 48 hr, but not with freshly isolated unstimulated lymphocytes, indicating that the absorption of IL-2 activity results from the binding of IL-2 molecules to IL-2 receptors on Con A-stimulated lymphocytes.—KEY WORDS: feline IL-2, IL-2 receptor.

Interleukin 2 (IL-2) is one of lymphokines from T cells stimulated with either specific antigens or lectins, and has many biological properties such as the induction of cytotoxic T cells [7], activation of natural killer cells [6] and enhancement of gamma-interferon production by T cells [7]. These findings suggest that IL-2 plays an important role in the regulation of cell-mediated immunity.

The alterations in the production of IL-2 [3, 16], the responsiveness to IL-2 and the expression of IL-2 receptors [20, 24] are found in patients with either adult T cell leukemia/lymphoma syndrome (AIDS). ATL and AIDS appear to be caused by human T cell leukemia/lymphoma virus type I and III, respectively, which are in a family of retrovirus like feline leukemia virus (FeLV) [14, 19]. FeLV-related diseases as well as leukemia/lymphosarcoma in the cat show many similarities in clinical and pathological features of ATL and AIDS [5, 15]. Thus, it is of great importance to define whether IL-2 is concerned with immunological abnormalities in cats with FeLV-related diseases or leukemia/lymphosarcoma. However, in comparison to a large number of researches of IL-2 in the rodent and human, few available information concerning feline IL-2 has been provided [12, 18].

Therefore, the purpose of the present study is to determine and to characterize feline IL-2 by using the murine IL-2 dependent T cell line (CTLL-2) cell proliferation assay.

MATERIALS AND METHODS

Animals: Normal eight adult cats (4 males and 4 females, ranging 2 to 8 years old) were used in the present study. No abnormal findings were obtained by hematological and biochemical examinations, urinalysis and fecal examination. FeLV group specific antigen was negative by an enzyme-linked immunosorbent assay (ELISA) using a Leukassay F kit (Pitman Moor Inc., Washington Crossing, NJ) and by an indirect immuno-
fluorescence test [13].

Six-week-old rats (Wistar-Imamichi strain) were used for the source of IL-2 to maintain an IL-2 dependent cell line described below.

Eight-week-old BALB/c mice were also used for the preparation of IL-2.

Culture media: RPMI medium 1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) (v/v), 25 μg/ml of gentamicin, and 0.1% sodium bicarbonate, referred to as complete medium (CM), was used. For the induction of IL-2 production and IL-2 receptor expression, 10 ng/ml phorbol myristate acetate (PMA; Sigma Chemical Co., St. Louis, MO), 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; Research Organic Inc., Cleaveland, OH), 5× 10^{-5} M 2-mercaptoethanol (2-ME; Wako Pure Chemical Industries, Japan) and an additional 1 mM of L-glutamine (Nissui Pharmaceutical Co., Japan) were added to CM.

Lymphocyte preparation: Lymphocytes from the spleens of cats, rats and mice were prepared according to the method described elsewhere [21].

Peripheral blood lymphocytes (PBL) were also obtained from heparinized blood (5–10 ml) of cats. Enriched lymphocyte fractions were separated by the method of Taylor et al. [23].

The resultant cell preparations contained more than 90% lymphocytes. The viability of cells was always more than 95%, as determined by the trypan blue (0.25%) dye exclusion test.

Preparation of IL-2 from splenic lymphocytes and PBL: Crude IL-2 preparations were made from concanavalin A (Con A; Sigma)-stimulated splenic lymphocytes from rats and mice, according to the method of Gillis et al. [8]. Feline IL-2 preparations were made by a minor modification of the procedure used for murine IL-2 preparations. Splenic lymphocytes (2×10^6 cells/ml) from cats were cultured with Con A (5 μg/ml) for 12 hr at 37°C in a 5% CO2-humidified atmosphere.

PBL (2×10^6 cells/ml) from healthy cats were incubated with Con A at varying concentrations of 1 to 100 μg/ml for 1 to 48 hr at 37°C in a 5% CO2-humidified atmosphere.

After the incubation, the supernatant fluids from splenic lymphocyte and PBL cultures were collected, filtered through 0.2 μm Millipore filters (Millipore Co., Bedford, MA), and stored at −20°C until used.

Partial purification of feline IL-2: Partial purification of feline IL-2 was carried out as described before [19].

Culture supernatants of splenic lymphocytes stimulated with Con A were brought to 85% saturation with solid ammonium sulfate by gentle stirring until dissolved at 4°C, kept at 4°C for at least 12 hr, and centrifuged at 8000 rpm for 20 min. The precipitate was suspended into the desired volume of 0.9% sterile saline and dialyzed against 100-fold volume of 0.9% sterile saline for 24 hr. The sample was applied to a Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden) packed column (2.5×90 cm) that had been equilibrated in 0.9% saline. The column was eluted with 0.9% sterile saline at a flow rate of 18 ml/hr at 4°C. The protein content of the eluted fractions (4 ml/tube) collected was recorded with the aid of a Mini UV monitor (Atto Co., Japan) calibrated with the following molecular weight (m.w.) standard markers (Pharmacia): blue dextran (exclusion marker, m.w. 2000000), bovine serum albumin (m.w. 67000), ovalbumin (m.w. 43000), chymotrypsinogen-A (m.w. 25000) and ribonuclease-A (m.w. 13700). Fractions (1 ml) were collected, diluted with CM and assayed for IL-2 activity. After the fractions containing IL-2 activity were pooled, polyethylene glycol 6000 (Wako) at a final concentration of 1 μg/ml was added to the pooled fractions to stabilize IL-2 activity [25]. For biochemical analysis, bovine serum albumin (Sigma) at a final concentration of 0.5 mg/ml was supplemented to an aliquot of the pooled
samples.

Chemical and temperature treatments of feline IL-2: The pooled feline IL-2 samples partially purified by column chromatography were dialyzed against 50 mM ammonium bicarbonate, aliquoted into 1 ml fractions, lyophilized and subjected to each treatment as follows. After the treatment each samples was dialyzed against phosphate buffered saline (PBS, pH 7.2) assayed for IL-2 activity and compared with the activity of control IL-2 samples resuspended in a buffer (PBS, pH 7.2).

Lyophilized IL-2 samples were resuspended in a Na2HPO4-citric acid buffer (pH 3.2), and a Na2CO3-NaHCO3 buffer (pH 10.5). IL-2 samples were incubated in each of different buffers for 4.5 hr at 4°C. The sensitivity of IL-2 activity to urea-induced denaturation was determined by incubating IL-2 samples in 2, 4 and 8 M urea (Kokusan Chemical Works Co., Japan) in PBS (pH 7.2) for 4.5 hr at room temperature. Feline IL-2 preparations were also incubated with trypsin (Worthington Diagnostic Systems Inc., Freehold, NJ) at the concentration of 100 μg/ml in 50 mM Tris buffer (pH 7.5) supplemented with 0.1 M CaCl2 and 0.1 M NaCl at 37°C for 12 hr. After trypsin treatment, heat-inactivated fetal bovine serum was added to a final concentration of 10% to stop enzymatic reaction.

Lyophilized feline IL-2 preparations were resuspended in PBS (pH 7.2) and incubated at 37°C for 12 hr, 56°C for 1 hr, and 70°C for 15 min, 30 min and 1 hr, respectively.

Assay for IL-2 activity: IL-2 activity in culture supernatants was assayed using an IL-2 dependent cloned murine cytotoxic T cell line (CTLL-2) as previously described [8]. This cell line has been maintained at an interval of 3 days. CTLL cells were washed twice to remove residual rat IL-2 and then seeded at 4×10⁴ cells/ml in each well of a 96-well flat-bottomed microtiter plate (Falcon 3072; Becton Dickinson Labware, Ox-}

The cells were cultured in the presence of each of culture supernatants, diluted finally to 1:8 unless described, for 24 hr at 37°C in a 5% CO2 humidified atmosphere. The cells were pulsed with 0.4 μCi of tritiated thymidine (³H-TdR; 6.7 Ci/mM, New England Nuclear, Boston, MA) 4 hr before terminating the cultures. Cells were then harvested onto glass fiber filters (Labo Science Co., Japan) with a semiautomatic multiple-sample harvester (Labo Science). The amount of incorporated radioactivity was measured in a liquid scintillation counter (Aloka Co., Japan) and the results were expressed as the mean and standard error (SE) of count per minute (cpm) for triplicate cultures.

Proliferative responses of feline PBL to partially purified feline IL-2: The assay for IL-2 mediated proliferative response of PBL was carried out, after the optimal proliferative response of PBL to Con A was determined. The proliferative response of PBL to Con A was performed according to the procedure described by Cockerell et al. [4]. Our preliminary experiment showed that Con A-induced lymphocyte proliferation was maximal when PBL (1×10⁶ cell/ml) were cultured with Con A (12.5 μg/ml) for 72 hr. This optimal culture condition was employed in the subsequent study.

For IL-2 mediated proliferative response, PBL (1×10⁶ cells/ml) were added into each well of a microtiter plate (Falcon 3072) and stimulated in the presence of 5% or 10% partially purified feline IL-2 preparations with or without Con A (12.5 μg/ml) for the period ranging from 24 to 96 hr at 37°C in a humidified atmosphere of 5% CO2 in air. The partially purified IL-2 preparations used in the present study showed to have a potent effect on CTLL cell proliferation (35000±2300 cpm) at the concentration of 10%. Eighteen hr before the termination of the cultures, 0.25 μCi of ³H-TdR were added to each well. After the culture, the amount of
incorporated radioactivity was measured by the same manner as described above. The results were expressed as the mean and SE of cpm for triplicate cultures.

**Estimation of feline IL-2 receptors:** An indirect estimation of IL-2 receptors on feline PBL was performed according to a minor modification of the method of Burger et al. [2], which is referred as the absorption test.

Freshly isolated PBL (2×10⁶ cells/ml) were cultured with Con A (10 µg/ml) for 48 hr at 37°C in a humidified atmosphere of 5% CO₂ in air. After incubation, the viable cells were washed twice with CM, adjusted to the desired cell concentrations and centrifuged at 1500 rpm for 10 min. Con A-stimulated or freshly isolated PBL were resuspended in 0.1 ml of partially purified feline IL-2 preparations and incubated for 4 hr at 4°C. After centrifugation at 1500 rpm for 10 min, the supernatant was harvested, and the residual IL-2 activity in serially diluted resultant supernatants was measured by the CTLL cell proliferation assay. The results were expressed as the mean cpm and a percent of that of control cultures in which CTLL cells were cultured in the presence of partially purified IL-2 preparations untreated with PBL.

**RESULTS**

**Proliferation of CTLL cells by the culture supernatants of splenic lymphocytes stimulated with Con A:** The proliferative response of CTLL cells by the supernatant from Con A-stimulated feline splenic lymphocytes was approximately similar to that from Con A-stimulated murine splenic lymphocytes, as shown in Fig. 1. The maximum proliferation was observed at the dilution of 1:4 or 1:8 of both culture supernatants from murine and feline splenic lymphocytes. The culture supernatant from unstimulated feline splenic lymphocytes failed to induce the CTLL cell proliferation at any dilutions.

**Estimation of m.w. of feline IL-2:** A high
Table 1. Effects of chemical and temperature treatments on feline IL-2 activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Response of CTL cells&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>³H-TdR incorporation (cpm)</th>
<th>% Decrease of IL-2 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>23770</td>
<td>0</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td></td>
<td>8977</td>
<td>62</td>
</tr>
<tr>
<td>10.5</td>
<td></td>
<td>5304</td>
<td>78</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 M</td>
<td></td>
<td>4249</td>
<td>82</td>
</tr>
<tr>
<td>4 M</td>
<td></td>
<td>5710</td>
<td>76</td>
</tr>
<tr>
<td>8 M</td>
<td></td>
<td>5696</td>
<td>76</td>
</tr>
<tr>
<td>Trypsin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µg/ml</td>
<td></td>
<td>949</td>
<td>96</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37°C (12 hr)</td>
<td></td>
<td>13977</td>
<td>41</td>
</tr>
<tr>
<td>56°C (1 hr)</td>
<td></td>
<td>15332</td>
<td>35</td>
</tr>
<tr>
<td>70°C (15 min)</td>
<td></td>
<td>798</td>
<td>97</td>
</tr>
<tr>
<td>70°C (30 min)</td>
<td></td>
<td>55</td>
<td>100</td>
</tr>
<tr>
<td>70°C (1 hr)</td>
<td></td>
<td>73</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup) Each assay was performed by using partially purified feline IL-2 preparations.

Fig. 3. IL-2 activity in culture supernatant of PBL stimulated with Con A. PBL (2×10⁶ cells/ml) were cultured for 1 to 48 hr with Con A of 1 µg/ml (*), 2.5 µg/ml (•), 5 µg/ml (○), 10 µg/ml (△), 50 µg/ml (▲) and 100 µg/ml (■). The IL-2 activity in the cell-free supernatants was measured by the CTL cell proliferation assay. Each point represents the mean cpm of ³H-TdR incorporation in triplicate cultures from three cats and SE shown by vertical bar.

A high level of IL-2 activity was present in the eluted fractions, ranging from the m.w. of approximately 13000 to 20000 daltons (Fig. 2). The relative m.w. of feline IL-2 was thus estimated to be approximately 16000 daltons, based on the median of these fractions containing IL-2 activity. A low level of IL-2 activity was also observed in higher m.w. fractions in the same elution profile.

Physico-chemical properties of partially purified feline IL-2 obtained by Sephadex G-100 gel filtration chromatography: The physico-chemical properties of partially purified feline IL-2 were tested and compared with those of untreated control preparations (Table 1). IL-2 activity was partially reduced after incubated with pH 3.2 and pH 10.5 buffers, and treated with urea (2, 4 and 8 M). The treatment of IL-2 preparations with trypsin also resulted in a remarkable decrease (96%) of its activity.

Feline IL-2 activity was not affected after incubated at 37°C for 12 hr and 56°C for 1 hr, but remarkably decreased by heating at 70°C for 15 min., 30 min and 1 hr, respectively.
of high concentrations (50–100 μg/ml), whereas IL-2 activity sharply decreased 48 hr after cultured with Con A of low concentrations (1–10 μg/ml).

**Proliferative response of feline PBL to partially purified feline IL-2 preparations:** The effect of the addition of feline IL-2 preparations on the proliferative response of PBL to ConA is presented in Fig. 4. The supplement with feline IL-2 preparations alone failed to induced the proliferative responses of lymphocytes. The supplement of feline IL-2 preparations of 5% or 10% resulted in enhanced proliferative responses (32000 cpm and 36000 cpm, respectively), as compared to those cultured with Con A alone (26000 cpm).

**Estimation of IL-2 receptors on feline lymphocytes:** For estimation of the generation of IL-2 receptors on feline PBL, IL-2 activity was measured after absorbed with either fresh or Con A-stimulated PBL.

The incubation of feline IL-2 preparations with Con A-stimulated PBL (5×10^9–2×10^9 cells) resulted in decreased levels (26–51%) of IL-2 activity. The supernatants after incubated with freshly isolated PBL, however, failed to induce significant decreases (1–11%) in IL-2 activity (Table 2).

**DISCUSSION**

In the present report, feline IL-2 activity and its physico-chemical properties were described.

The IL-2 dependent murine T cell line (CTLL-2) has been widely used for the determination of IL-2 activity in humans and rodents. The culture supernatant of feline splenic lymphocytes stimulated with Con A contained a high level of biological activity promoting the growth of CTLL cells, which is comparable to those of murine IL-2 preparations. This result indicates that feline IL-2, like human and murine IL-2, can be demonstrated by the CTLL cell proliferation assay.
Table 2. Absorption of IL-2 activity by freshly isolated lymphocytes and Con A-stimulated lymphocytes

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Response of CTL cellsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells for absorption</td>
<td>No. of cells</td>
</tr>
<tr>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Freshly isolated lymphocytes</td>
<td>5×10^5</td>
</tr>
<tr>
<td></td>
<td>1×10^6</td>
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<tr>
<td></td>
<td>2×10^6</td>
</tr>
<tr>
<td>Con A-stimulated lymphocytes</td>
<td>5×10^5</td>
</tr>
<tr>
<td></td>
<td>1×10^6</td>
</tr>
<tr>
<td></td>
<td>2×10^6</td>
</tr>
</tbody>
</table>

a) Freshly isolated PBL or PBL stimulated with Con A (10 μg/ml) for 48 hr were incubated with feline IL-2 preparations for 4 hr at 4°C. Each value represents cell numbers/0.1 ml.
b) IL-2 activity remaining in feline IL-2 preparations after a 4-hr incubation with freshly isolated PBL or Con A-stimulated PBL is indicated in the mean cpm incorporated in triplicate cultures and % control IL-2 activity.

Therefore, the CTL cell proliferation assay system appeared to be available for determining feline IL-2 activity.

Sephadex G-100 gel filtration chromatography study revealed that feline IL-2 activity was present mainly in the fractions equivalent to a relative m.w. ranging from 13000 and 20000 daltons. Based upon the median of these fractions containing IL-2 activity, the m.w. of feline IL-2 was estimated to be approximately 16000 daltons, which closely resembles those of human and rat IL-2 [10]. A low level of IL-2 activity was also present in higher m.w. fractions in the same chromatographic profile. This may be attributed to the complexes formed by aggregated IL-2 molecules, as suggested by Gillis et al. [10]. To investigate this possibility, the high m.w. fractions (fraction Nos. 35–45) containing IL-2 activity were pooled, concentrated, dialyzed against 0.5 M NaCl and rechromatographed on the same Sephadex G-100 column. Almost all parts of activity in the higher m.w. fractions then disappeared and a low level of IL-2 activity was observed in the fraction equivalent to the m.w. of approximately 16000 daltons.

Feline IL-2 showed some physical and biochemical properties similar to human and murine IL-2, as shown to be sensitive to trypsin treatment and to be labile to heating at 70°C. Feline IL-2 was rather sensitive to the incubation at pH 3.2 and pH 10.5, and the treatment with urea (2, 4 and 8 M), as compared to those of human [9] and murine [17] IL-2.

The kinetics of feline IL-2 production showed that the maximal level of IL-2 activity in the culture supernatant appeared as early as 6–12 hr after incubated with Con A (10–100 μg/ml). This result on the kinetics of feline IL-2 production was slightly different from those on rat [8] and murine [11] IL-2.

The addition of feline IL-2 to lymphocyte cultures containing Con A resulted in the enhanced lymphocyte proliferation, whereas IL-2 alone in the absence of Con A failed to induce a significant lymphocyte proliferation. These results suggest that the proliferative response to IL-2 requires the activation of lymphocytes with Con A [22]. This was also confirmed on the basis of the findings obtained by the absorption test; IL-2 activity was remarkably absorbed only with Con A-stimulated lymphocytes, but not with freshly isolated lymphocytes. These findings, there-
fore, suggest the generation of IL-2 receptors on Con A-stimulated feline lymphocytes [1].

The method described here can provide useful means of evaluating IL-2 production by T cells and their responsiveness to IL-2, and of analysing lectin-induced IL-2 receptors on lymphocytes to assess lymphocyte functions as one of parameters of cell-mediated immune responses in the cat.

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REFERENCES


要 約

ネコのインターロイキン2活性：後藤塚 像・鶴田好和・長谷川篤彦・友田 勇（東京大学農学部家畜内科学教室）——ネコリンバ球をconcanaevalin A（Con A）とともに培養した上清中にマウスのIL-2依存性T細胞株（CTLL-2）を増殖させる活性が検出され、ネコ IL-2活性を CTLL細胞の増殖反応によって測定できることが明らかになった。ネコ IL-2活性は、ゲル過法により分子量約16000ダルトンに相当する分画に存在することが判明し、ネコ IL-2の分子量もヒト及びラット IL-2のそれに類似することが示された。本ネコ IL-2分画を用いて、そのいくつかの物理化学的性状について検討した結果、ネコ IL-2はトリプシン処理によって失活し、酸（pH 3.2）、アルカリ（pH 10.5）、熱（70℃）及び尿素（2, 4, 8M）処理に対してもヒトやマウス IL-2に比較して感受性が高いことが確認された。Con Aで刺激したリンバ球培養上清中のIL-2活性は6～12時間後に最高値を示し、添加するCon A濃度は、10～100 μg/mlで最大のIL-2活性が得られた。次いでネコリンバ球培養におけるIL-2の添加効果について検討した。その結果、ネコ IL-2のみを添加した場合にはネコリンバ球の増殖はおこらなかったが、Con Aの存在下においてネコ IL-2を添加した場合には増殖が促進された。またIL-2をCon Aで刺激していないリンバ球で処理しても IL-2活性の有意的な減少は認められなかったが、Con Aで刺激したリンバ球で処理した場合には著明な減少が認められた。このことから、ネコリンバ球をCon Aで刺激すると IL-2レセプターが発現するものと考えられた。