Release of Interleukin 1 from Peritoneal Exudate Cells of Cats with Feline Infectious Peritonitis

Ryo GOITSUKA, Yoshikazu HIROTA, Atsuhiro HASEGAWA, and Isamu TOMODA
Department of Veterinary Medicine, Faculty of Agriculture, University of Tokyo, Tokyo 113, Japan

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ABSTRACT. A significant amount of mouse thymocyte proliferation promoting activity was detected in the culture supernatants and cell lysates of peritoneal exudate cells (PEC) from cats with spontaneously occurring effusive feline infectious peritonitis (FIP). The production of the thymocyte proliferating factors appeared to be independent of the stimulation with lipopolysaccharide (LPS). In contrast to PEC, peripheral blood leukocytes from cats with FIP failed to release a high level of thymocyte proliferating activity without LPS stimulation, indicating that the cells localized in inflammatory sites were activated to release the biological active factors. Thymocyte proliferating activity in the culture supernatants of PEC from cats with FIP was inactivated by heating at 70°C for 30 min. The molecular weight of the active factors was approximately 12000–20000 daltons with Sephacryl S-200 gel filtration, and the isoelectric points of this activity were 4.1, 4.8, 5.3 and 6.8, respectively, with chromatofocusing. These results indicate that the thymocyte proliferation observed here is attributed to feline interleukin 1 (IL-1). Therefore, the release of IL-1 by cells activated in inflammatory sites might be at least in part involved in the pathogenesis of vascular lesions seen in cats with effusive FIP.—KEY WORDS: feline infectious peritonitis, IL-1, peritoneal exudate cell.

Interleukin 1 (IL-1) has been shown to induce the adhesion of leukocytes to vascular endothelial cells [4], to induce tissue factor like procoagulant activity [3], and to promote leukocyte chemotaxis [10]. These biological properties have been supposed to be involved in the pathogenesis of several vascular diseases [16, 21].

In the context of these vascular diseases, coronavirus-induced systemic vasculitis has been noted in cats with feline infectious peritonitis (FIP). This disease is characterized by distinct features including fibrositis with the accumulation of effusive fluids and/or disseminated granuloma formation in various organs. These lesions are associated with polyvasculitis, accompanied by intense infiltrations of neutrophils and to a lesser extent macrophages and lymphocytes [30]. The pathogenesis of FIP is suggested to be immune mediated, as supported by several findings; 1) the enhanced development of FIP in the presence of antibodies against FIP virus [23], 2) the presence of immune complexes in blood [15], and 3) the deposition of immune complexes in the renal glomeruli [13]. Therefore, it is of interest to study whether IL-1 is concerned with the pathogenesis of FIP.

The present study deals with the production of IL-1 by peritoneal exudate cells (PEC) from cats with effusive FIP. Furthermore, IL-1 like factors detected in PEC cultures were analyzed for several physicochemical properties including heat sensitivity, molecular weight (m. w.) and isoelectric point (pI).

MATERIALS AND METHODS

Animals: Thirteen cats ranging from 3 months to 3 years old, admitted to the Veterinary Hospital, University of Tokyo,
with effusive FIP, were used in the present study. The definitive diagnosis of FIP was made on the basis of the following findings in these cats: 1) high serum titers of antibody against FIP virus (greater than 1:400), as determined by the indirect immunofluorescent antibody test previously described by Hayashi et al. [14], 2) accumulation of ascitic fluid, and 3) hypergammaglobulinemia. In addition, cytopathic virus isolated from two cats (case Nos. 4 and 9) by using a coculture technique of PEC and feline macrophage-like cell line (fcwf-4) cells, was confirmed to be coronavirus by immunofluorescence using anti-FIP virus serum from a FIP cat and by electron microscopy. Immunological evaluations were all carried out before therapy.

C3H/HeJ mice ranging from 4 to 6 weeks old (Jackson Laboratory, Bar Harbor, ME) free of mouse hepatitis virus, were also used to determine IL-1 activity.

Cell preparations: For PEC preparation, ascites were collected from cats with effusive FIP, and centrifuged at 1500 rpm for 10 min. The resulting pelleted cells were washed twice with phosphate buffered saline (PBS), and suspended at a concentration of $2 \times 10^6$ cells/ml in RPMI 1640 medium (Gibco; Grand Island Biological Co., NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 25 $\mu$g/ml of gentamicin, hereafter referred to as medium. The PEC were depleted of macrophages by means of surface adherence to plastic dishes. Giemsa-stained smears of the cell suspensions were then prepared for cytological examination.

Peripheral blood leukocytes were also obtained from the heparinized blood of cats with FIP. Theuffy coat layer was treated with 0.8% ammonium chloride to remove erythrocytes, washed twice with PBS, and resuspended in medium at a concentration of $2 \times 10^6$ cells/ml.

Supernatant preparations: Each of PEC, macrophage-depleted PEC, and peripheral blood leukocytes were cultured at $2 \times 10^6$ cells/ml for 24 hr in the presence or absence of LPS (E. coli serotype 0.55:B5, Difco Laboratories, Detroit, MI) at a concentration of 10 $\mu$g/ml at 37°C in a 5% CO$_2$-humidified atmosphere. Each culture supernatant was centrifuged at 3000 rpm for 10 min. The resulting cell-free supernatant fluids were harvested, and stored at −20°C until used.

PEC lysates were obtained by sonifying PEC ($1 \times 10^7$ cells/ml) for 5 min in a sonicator (Sanko Junyaku Co., Japan). The lysates were spun at 3000 rpm for 10 min to remove cell debris, and the resultant supernatants were then Millipore-filtered and stocked at −20°C until tested.

Heat treatment: The culture supernatants of unstimulated PEC from cats with FIP were incubated at various temperatures for 30 min, and then assayed for thymocyte proliferating activity.

Gel filtration and chromatofocusing: Before gel filtration, the culture supernatants prepared from PEC were concentrated 10 fold by vacuum dialysis. The concentrated samples (2 ml) were applied to a calibrated column (2.5×90 cm) of Sephacryl S-200 (Pharmaica Fine Chemicals, Uppsala, Sweden) equilibrated with PBS, and then were eluted with PBS at a flow rate of 20 ml/hr. Fractions of 4 ml were collected, and assayed for thymocyte proliferating activity.

Fractions containing thymocyte proliferating activity after Sephacryl S-200 gel filtration were pooled, dialyzed against 0.025 M imidazole-HCl buffer, pH 7.4, and then applied to a 1×21 cm chromatofocusing column of Polybuffer Exchanger 94 (PBE 94; Pharmacia). The samples were eluted with Polybuffer 74 (Pharmacia), pH 4.0, at a flow rate of 20 ml/hr. Fractions of 4 ml were collected. Each sample was dialyzed against PBS, and then assayed for thymocyte proliferating activity.
Table 1. Cellular composition of ascites from cats with FIP

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Cell number (μl)</th>
<th>Differential percent</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PMN</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>1</td>
<td>160</td>
<td>90</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>6250</td>
<td>78</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>2290</td>
<td>53</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>140</td>
<td>75</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>73</td>
<td>11</td>
</tr>
<tr>
<td>6</td>
<td>1320</td>
<td>97</td>
<td>1</td>
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<tr>
<td>7</td>
<td>180</td>
<td>80</td>
<td>5</td>
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<tr>
<td>8</td>
<td>1750</td>
<td>96</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>4900</td>
<td>86</td>
<td>9</td>
</tr>
<tr>
<td>10</td>
<td>1710</td>
<td>77</td>
<td>8</td>
</tr>
<tr>
<td>11</td>
<td>630</td>
<td>11</td>
<td>45</td>
</tr>
<tr>
<td>12</td>
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<td>32</td>
<td>6</td>
</tr>
<tr>
<td>13</td>
<td>4250</td>
<td>78</td>
<td>9</td>
</tr>
</tbody>
</table>

a) The relative proportion of PEC after counting minimum 200 cells on each Giemsa-stained PEC smear is given.

**Assay for thymocyte proliferating activity:** Thymocyte proliferating activity was measured by the method of Mizel *et al.* [20]. Briefly, thymocytes (1.5 × 10⁶ cells) from C3H/HeJ mice were placed into each well of 96-well flat-bottomed microtiter plates (Falcon 3072; Becton Dickinson Labware, Oxnard, CA) and cultured with 1 μg/ml of phytohemagglutinin (PHA; Wellcome Foundation, Ltd., Beckenham, England) in the presence or absence of various dilutions of test samples. The cells were pulsed with 0.4 μCi of tritiated thymidine (³H-TdR; 6.7 Ci/mM, New England Nuclear, Boston, MA) per well for 8 hr before harvesting onto glass-fiber filters. The amount of incorporated radioactivity was quantified by liquid scintillation counting. The results were expressed as the mean of count per minute (cpm) for triplicate cultures.

**Assay for IL-2 activity:** IL-2 assay was performed as described earlier [7, 8]. Briefly, murine IL-2 dependent T cell line (CTLL-2) cells (4 × 10³ cells) were cultured for 20 hr in microtiter wells containing serially diluted test samples at 37°C in a 5% CO₂-humidified atmosphere. The cells were pulsed for 4 hr with 0.4 μCi of ³H-TdR per well, and harvested onto glass fiber filters. ³H-TdR incorporation was assessed by liquid scintillation counting.

**RESULTS**

The numbers and cellular compositions of the PEC from 13 cases of spontaneous effusive FIP are summarized in Table 1. The number of PEC varied from 100 to 6250 cells/μl. Cytological evaluation of the PEC revealed relative proportions of 71.2% polymorphonuclear cells (PMN) (ranging from 11 to 97%), 18.3% macrophages (2 to 62%) and 10.5% lymphocytes (1 to 45%).

All of the culture supernatants of PEC
Table 3. Thymocyte proliferating activity in the culture supernatants of unfractonated and non-adherent PEC from cats with FIP

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Unfractionated</th>
<th>Non-adherent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12375</td>
<td>14118</td>
</tr>
<tr>
<td>2</td>
<td>4398</td>
<td>16152</td>
</tr>
<tr>
<td>3</td>
<td>14750</td>
<td>11907</td>
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<tr>
<td>8</td>
<td>2323</td>
<td>4180</td>
</tr>
<tr>
<td>10</td>
<td>1873</td>
<td>3087</td>
</tr>
</tbody>
</table>

a) The non-adherent cell fraction of PEC from cats with FIP was obtained by adherence of PEC to plastic dishes. Unfractionated PEC and non-adherent PEC were cultured for 24 hr without any stimulation. The resulting cell-free supernatants were harvested and assayed for thymocytes proliferating activity. The results indicate $^3$H-TdR incorporation (mean cpm) by thymocytes cultured with 4-fold diluted culture supernatants from each PEC for 72 hr. Background incorporation induced by PHA alone was 1565 cpm.

After a 24-hr incubation without any stimulation contained PHA-induced mouse thymocyte proliferation promoting activity (Table 2). In 6 out of 13 FIP cats, unstimulated PEC spontaneously released more thymocyte proliferating factors than did LPS-stimulated PEC. In contrast, the stimulation of PEC with LPS resulted in substantially increased levels of the activity in 2 cats, as compared with those spontaneously released by unstimulated PEC.

The cell lysates of PEC from 2 cats with FIP (case Nos. 2 and 3) had higher levels of thymocyte proliferating activity (9220 and 10261 cpm, respectively), as compared to background incorporation induced by PHA alone (1969 cpm).

No IL-2 activity was found in these culture supernatants of PEC, as determined by the CTL assay. In addition, virus preparations from FIP virus-infected fowl cells did not affect PHA-induced mouse thymocyte proliferation.

Cell suspension of unfractonated PEC and of nonadherent PEC depleted of macrophages by surface adherence to plastic dishes (less than 3% macrophages) were cultured for 24 hr without any stimulation, and then the resulting cell-free supernatants were assayed for thymocyte proliferating activity. The activity in the culture supernatants from non-adherent PEC was higher than or comparable with that observed in unfractonated PEC (Table 3).

The ability to release thymocyte proliferating factors was evaluated in peripheral blood leukocytes from 4 cats with FIP. The relative proportions of these cells were 93% PMN (ranging from 84 to 99%) and 7% lymphocytes (ranging from 1 to 16%). The culture supernatants of peripheral blood leukocytes had a significantly lower level of thymocyte proliferating activity than that of PEC (Table 4).

The thymocyte proliferating activity in the culture supernatants of PEC from cats with FIP was partially reduced by heating at 56°C for 30 min, and completely destroyed by heating at 70°C for 30 min (Table 5).

A representative Sepharcl S-200 elution profile of thymocyte proliferating factors derived from PEC cultures of cats with FIP is shown in Fig. 1. Thymocyte proliferating activity was detected in the fractions with a
m. w. ranging from 12000 to 20000 daltons. The active fractions were pooled, and then subjected to chromatofocusing. Thymocyte proliferating activity was eluted into 4 different components with pIs of 4.1, 4.8, 5.3 and 6.8, respectively, as shown in Fig. 2.

DISCUSSION

IL-1 production by monocytes and several

Table 5. Effects of heat treatment on thymocyte proliferating activity in the PEC culture

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^3$H-TdR incorporation$^a$ (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>6173</td>
</tr>
<tr>
<td>Heated at 56°C for 30 min</td>
<td>1463</td>
</tr>
<tr>
<td>Heated at 70°C for 30 min</td>
<td>710</td>
</tr>
</tbody>
</table>

$^a$ $^3$H-TdR incorporation (mean cpm) by thymocytes cultured with 4-fold diluted culture supernatants of PEC from cats with FIP for 72 hr is given. Background incorporation induced by PHA alone was 814 cpm.

Fig. 1. Gel filtration of thymocyte proliferating factors released by PEC from cats with FIP on Sephacryl S-200. Two millilitres of 10-fold concentrated samples were applied to the column which had been calibrated with the following m. w. markers; bovine serum albumin (67K), ovalbumin (43K), chymotrypsinogen (25K), myoglobin (17K) and cytochrome C (12.5K). Fractions of 4 ml were collected, and assayed for thymocyte proliferating activity at a 1:4 dilution in the presence of PHA.

Fig. 2. Chromatofocusing of thymocyte proliferating factors released by PEC from cats with FIP. Fractions containing thymocyte proliferating activity after Sephacryl S-200 gel filtration were pooled and dialyzed against imidazole-HCl buffer, pH 7.4. The samples were then applied to a PBE 94 chromatofocusing column and eluted into 4 ml fractions with a continuous gradient of pH 7.4 to 4.0 by using Polybuffer 74, pH 4.0, at a flow rate of 20 ml/hr. Each fraction was measured for its pH, dialyzed against PBS, and then tested for thymocyte proliferating activity at a 1:4 dilution in the presence of PHA.
peripheral blood leukocytes from cats with FIP failed to release a detectable level of thymocyte proliferating activity, suggesting a dissociation between local and systemic immune responses.

Although monocyte/macrophages are the main sources of IL-1 [25], PMN [22, 28], B cells [18], and T cells [27] also proved to release IL-1. From our results that the depletion of adherent cell populations in PEC had no effect on the accumulation of thymocyte proliferating activity, it was supposed that non-adherent cell populations such as PMN and lymphocytes as well as adherent cell populations might release thymocyte proliferating factors.

The results of heat sensitivity and the relative m. w. indicate that the thymocyte proliferation induced by the culture supernatants of PEC from cats with FIP is attributable to biologically active feline IL-1 [9].

Isoelectric point analysis revealed four peaks of IL-1 activity in the fractions corresponding to pIs 4.1, 4.8, 5.3 and 6.8, respectively. The active fraction with pI 6.8 occupied a comparable amount of IL-1 released by PEC from cats with FIP, whereas the predominant forms of IL-1 produced by LPS-stimulated feline alveolar macrophages from healthy cats were pIs 4.1, 4.8 and 5.3, as previously described [9]. Recent cloning of pI 5 (IL-1α) and pI 7 (IL-1β) forms of human IL-1 have demonstrated that these two pIs represent two distinct gene products [2, 17]. Hence, the pI 6.8 form of IL-1 released by PEC from cats with FIP seems to be comparable to the pI 7 form of human IL-1. However, it remains unclear whether the types of IL-1 predominantly produced are based on the cell types and functional state of IL-1 producing cells, or on the stimuli for the induction of IL-1. Simon and Willoughby [25] have demonstrated that LPS-stimulated rabbit alveolar macrophages produce IL-1 with two types of pIs 5 and 7, whereas LPS-stimulated alveolar macrophages from rabbits injected with bacillus Calmette-Guérin produce predominantly the pI 5 form of IL-1, implying that the type of isoelectric points of IL-1 produced is affected by the activation state of IL-1 producing cells. On the other hand, murine monocyte derived IL-1 has been reported to have only one form of IL-1 with pI 5 [19] despite of the recent cloning of the murine IL-1 gene equivalent to the human IL-1β gene [11]. Furthermore, murine PMN-derived IL-1 like factors have a pI 9 form as well as a pI 5 form [22]. These findings concerning murine IL-1 suggest that different types of cells produce distinct charges of IL-1. We are under investigation on the cell origin of IL-1 observed in the present study.

The production of IL-1 by PEC may contribute to characteristic clinicopathological features observed in cats with FIP including chronic fever, hypergammaglobulinemia, and pyogranulomatous inflammation around vessels, since IL-1 has been demonstrated to have a variety of functions to produce fever [6], to augment antibody production [26], and to promote the adhesion of leukocytes to vascular endothelial cells [4]. The spontaneous release of IL-1 in cats with FIP is possibly triggered by FIP virus infection. Furthermore, the enhancement of virus infection in phagocytic cells by non-neutralizing antibody might be associated with the pathogenesis of FIP, as in human cases of dengue fever [12]. Virus-antibody complexes as well as virus by itself [24] may have a potent effect on IL-1 production, as reported previously [1]. Therefore, further studies on IL-1 are required for more detailed understanding of the pathogenesis of FIP.

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REFERENCES


