Changes in Lymphokine-activated Killer Activity in Peripheral Blood Lymphocytes from Canine Transmissible Venereal Sarcoma Models

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Abstract: Time course changes in anti-tumor activity induced in peripheral blood lymphocytes (PBL) with recombinant human interleukin-2 (rhIL-2) and phytohemagglutinin-P (PHA) were studied in dogs implanted with canine transmissible venereal sarcoma (CTVS) as a tumor-bearing model. The rhIL-2-dependent and PHA-dependent cultures allowed selective proliferation of lymphocytes expressing Thy-1 antigens. The lymphocytes acquired a prolonged anti-tumor activity against the CTVS cells, starting from 2 weeks after the culture, indicating generation of lymphokine-activated killer (LAK) cells. The LAK cells showed serial growth in rhIL-2-containing culture medium for at least a further 2 weeks without loss of the anti-tumor activity.

Key words: CTVS, LAK cells, tumor model

Lymphokine-activated killer (LAK) cells were found as interleukin-2 (IL-2)-induced oncolytic lymphocytes by Rosenberg and his colleagues [2]. In brief, they demonstrated that peripheral blood lymphocytes (PBL) incubated in vitro with IL-2 for several days acquired cytotoxic activity (so called LAK activity) against not only natural killer (NK) cell-sensitive tumor cells but also NK cell-resistant tumor cells in a major histocompatibility complex (MHC)-unrestricted manner. In addition, they confirmed that the LAK cells showed in vivo cytotoxic activity in a murine tumor model [9], but no LAK activity in IL-2-dependent proliferating cells is observed in patients in the advanced stage of cancer [7, 10].

Canine transmissible venereal sarcoma (CTVS) is a spontaneously occurring tumor that affects the external genitalia of dogs, and is transmitted among dogs via coitus [1, 6]. This tumor can be induced experimentally in other dogs by inoculating with the living tumor cells, and is probably the only graft transplantable by means of allogeneic cells [1, 6]. The CTVS-bearing dogs are known to show signs of suppressed immune response as in a man with a malignant tumor, because the proliferative response of PBL obtained from the CTVS-bearing dogs to the tumor cells was suppressed to a significant level [4]. We therefore hypothesized

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that the CTVS-bearing dogs may be a valuable tumor model for studying \textit{in vitro} LAK cell functions (i.e., proliferative and cytotoxic activity) in cancer patients and that determination of the time course changes in LAK activity, induced in PBL obtained from the model, may make it possible to estimate the total number of the potent LAK cells generated in a culture period. The purpose of this study is to determine the onset and duration periods of LAK activity in PBL obtained from CTVS-bearing dogs, used as a model for human patients affected with neoplastic disease.

In the present study, 3 healthy beagle dogs (2 male and 1 female) were used for transplantation of CTVS cells, according to the method described by Koike \textit{et al.} [6]. In brief, transplantation was performed by subcutaneous injection of $10^6$ living CTVS cells to hypogastric lesions in each dog. Palpable nodules of CTVS were detected in all injection sites in all 3 dogs within a week after transplantation, and grew to the size of a hen-egg or a fist within 2–3 months. These dogs were therefore referred to as the tumor-bearing dogs (Fig. 1).

We then attempted to induce IL-2-dependent proliferating lymphocytes from PBL of the tumor-bearing dogs. In brief, canine PBL were isolated from heparin-treated blood by Ficoll-Conray density gradient centrifugation [8]. For the induction of canine LAK cells, RPMI-1640 (Gibco, Grant Island, USA) supplemented with 20 mM HEPES (Wako, Osaka), 100 U/ml penicillin G (Meiji, Tokyo), 100 µg/ml streptomycin (Meiji) and 10% horse serum (prepared in our laboratory) were used as the culture medium (CM). After washing the PBL 3 times with 0.01M phosphate-buffered saline (PBS; pH 7.4) the number of cells was adjusted to $2 \times 10^6$ cells/ml in CM, and the PBL were then stimulated with 10 µg/ml phytohemagglutinin-P (PHA; Difco, Detroit, USA) at 38°C in an atmosphere of 5% CO$_2$ for 48 hr in order to induce IL-2 receptor expression, as described previously [3]. PHA-stimulated cells were washed with PBS, resuspended to $2 \times 10^5$ cells/ml in CM and pulsed with 30–100 U/ml recombinant human IL-2 (rhIL-2; donated by Dr. M. Hattori, Kyoto University). PHA-stimulated cells proliferated in rhIL-2-containing CM, and the cell growth was supported by passage culture by means of repeated stimulations with fresh rhIL-2 (every 2–3 days) and PHA (every 3 passages) (Fig. 2). The doubling time of growth of the rhIL-2-dependent proliferating cells was estimated at 27.3 ± 9.3 hr (mean ± SD, n=33).

The smear preparations of the cultured cells of each

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig1.png}
\caption{A dog subcutaneously injected with CTVS cells into hypogastric lesions 2 months previously. CTVS has developed to the size of a hen-egg in the dog used as a model for tumor-bearing hosts.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{fig2.png}
\caption{Serial growth of LAK cells obtained from a CTVS-bearing dog. The cell growth is maintained by passage culture using repeated stimulations with rhIL-2 (every 2–3 days) and PHA (every 3 passages).}
\end{figure}
passage were fixed in formaldehyde acetone buffered solution (pH 6.6) at 4°C for 10 min, counter-stained with Giemsa solution, and observed under a light microscope. Some preparations were phenotypically analyzed immunocytochemically with ABC kits (Vectastain kit; Vector Lab., Burlingame, USA) [8]. Primary antibodies used as cell markers were as follows: rabbit IgG to dog IgG+IgM (Bethyl, Montgomery, USA), mouse monoclonal antibody (MoAb) to dog Thy-1 molecule (Serotec, Oxford, UK), and MoAb to granulocytes/monocytes (VMRD, Pullman, USA) served as markers of B cells, T cells and granulocytes/monocytes, respectively. The rhIL-2-dependent proliferating cells were mainly composed of large lymphoblastoid cells, and small- to middle-sized lymphocytes were observed scattered among the lymphoblastoid cells (Fig. 3). Most of the lymphocytes expressed Thy-1 antigens (Table 1), suggesting that the lymphocytes originate in cells of the T cell lineage(s).

In order to determine the onset and duration of LAK activity in the rhIL-2-dependent proliferating lymphocytes, we examined anti-tumor activities against CTBS cells in the lymphocytes incubated for 7, 14 and 28 days, and compared these data with those obtained from fresh PBL. In brief, 2 ml of CTBS cell suspension (cell density: $2 \times 10^6$ cells/ml) was mixed with an equal volume of lymphocyte suspension (cell density: $5 \times 10^6$ cells/ml) in a culture flask (Iwaki-Corning, Tokyo), and the co-culture was incubated at 38°C for 24 hr. To evaluate anti-tumor activity, the viability of the CTBS cells, used as target cells, was determined by trypan blue dye exclusion test [8]. In the test, lowered viability of the target CTBS cells means that lymphocytes, used as effectors cells, acquired cytotoxic activity (i.e., LAK activity), because our preliminary experiment revealed that the results obtained in the exclusion test correlated well with data obtained in the $^{31}$Cr release assay (data not shown). Fresh PBL or lymphocytes treated with rhIL-2 for 7 days appeared to have little effect on CTBS cells, whereas lymphocytes after 14 or 28 days of culture in the presence of rhIL-2 showed signs of significant anti-tumor activity against the CTBS cells (Fig. 4). These findings indicate that rhIL-2-dependent proliferating cells acquired LAK activity from at least 2 weeks after the culture, and that the LAK activity was sustained for more than 2 weeks. In our preliminary experiment and previous studies [2, 5], LAK cells, generated with IL-2 alone, had a peak of anti-tumor activity 4–7 days after incubation, whereas cells generated with PHA and IL-2 had a prolonged activity, starting from 14 days after the incubation. In brief,

![Fig. 3](image-url)  
Fig. 3. Light microscopic features of LAK cells (Giemsa stain, ×550). The cells are mainly large lymphoblastoid cells (arrow heads), and small- to middle-sized lymphocytes are also observed among the lymphoblastoid cells.

Table 1. Phenotypes of rhIL-2-dependent proliferating cells.

<table>
<thead>
<tr>
<th>Surface antigen</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thy-1</td>
<td>56.5 ± 12.5</td>
<td>94.5 ± 2.5</td>
<td>&gt;98</td>
<td>&gt;98</td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>25.0 ± 7.5</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>G/M</td>
<td>18.5 ± 5.5</td>
<td>2.2 ± 2.0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

a: granulocyte/monocyte markers. b: not detected.
LAK cells would undergo about a several thousand-fold expansion within 2 weeks. This method is therefore considered to be suitable for preparing large number of potent LAK cells. Interestingly, the LAK cells showed cytotoxicity against not only the CTVS cells but also PBL obtained from MHC-unmatched dogs (data not shown). The LAK cells may therefore include responder cells to tumor-specific antigens or allogeneic stimuli, and it remains unknown whether the LAK cells recognized the CTMS cells through tumor-specific antigens or allogeneic antigens on the tumor cells.

In conclusion, we were able to determine the time course changes in LAK activity, induced into PBL from CTMS-bearing dogs with rhIL-2 and PHA, by the trypan blue dye exclusion method. Further analysis of LAK cell functions in this tumor model would provide information on the induction of potent LAK cells from human patients with advanced cancer.

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