Establishment and Characterization of a T-Cell Line Specific for
Rickettsia tsutsugamushi

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To study the immunological protective system against rickettsial infection, a T-cell line specific for Rickettsia
tsutsugamushi antigen was established by long-term culture of splenocytes from mice immunized with live
Gilliam strain R. tsutsugamushi and then propagated in the presence of homologous rickettsial antigen and
syngeneic filler cells. The characteristics of the T-cell line and its capacity to induce antirickettsial protection
in vivo were studied. Flow cytometric analysis demonstrated that the T-cell line showed the phenotype Thy-1.2+
L3T4+ Lyt-2*, suggestive of helper T cells. In a lymphocyte proliferation assay, this cell line showed a specific
response to Gilliam antigen, partial cross-reactivity to Karp antigen, but no response to Kato antigen. The
proliferative response of this T-cell line was filler cell dependent, and genetic restriction was observed between
the T-cell line and filler cells. The T-cell line produced gamma interferon, one of the macrophage-activating
factors, in cultures with specific antigen and was able to adoptively mediate antirickettsial protection in vivo.
The data presented here suggest that antigen-specific helper T cells play an important role in protection against
rickettsial infection.

Rickettsia tsutsugamushi, the causative organism of tsutsugamushi disease, is known to be an obligate intracel-
lar microorganism. The protective mechanism of the host against rickettsial infection consists of a complex mutual
interaction of cell-mediated and humoral factors, in which cell-mediated immunity has been reported to play a particu-
larly important role (3, 5). The importance of the T-cell response in protection against rickettsial infection has been
proved by the following observations. (i) Athymic (nude) mice exhibit high susceptibility to a low-virulence strain of
R. tsutsugamushi, which is not pathogenic for eumycemic mice, and have died despite tetracycline treatment (9, 13).
(ii) By transfer of sensitized splenocytes or T cells, acquisition of protective immunity against infection can be ob-
served, and the protective effect is lost when the cells are treated with anti-Thy serum and complement (9, 13, 29).
(iii) Lymphocyte proliferative response to the rickettsial antigen in vitro is found after rickettsial infection with R. tsus-
tugamushi or R. rickettsii (4, 10). The mechanism of protection against rickettsial infection has also been gradually eluci-
dated since it has been reported that lymphokines produced by sensitized T cells activate macrophages to be ricket-
tsicidal (20, 21) and inhibit rickettsial growth in nonphagocy-
tic cells (32). However, the characteristics of T cells participating in this protective immunity have not yet been
fully elucidated and it was not clear whether all of these activities were carried out by a single population or by
several populations of T cells. The recent progress of cultivation techniques has made it possible to culture normal T
cells for long periods and analyze the mechanism of cell-
mediated immunity by using T-cell lines (11, 31).

In the present study, we established a T-cell line specific for rickettsial antigen by using the above-mentioned meth-
ods. Furthermore, we examined the properties, lymphokine production, and protective effect against infection of this
T-cell line.

MATERIALS AND METHODS

Mice. Female BALB/c, C57BL/6, and NZB mice at 8 to 12 weeks of age were used in all experiments.
Rickettsiae. The Gilliam, Karp, and Kato strains of R. tsutsugamushi, which have been previously described (14),
were used. Spleens were harvested from BALB/c mice heavily infected with each strain. They were then homoge-
nized into a 33% emulsion with brain heart infusion broth in a Waring blender and stored at −80°C until use. When
needed, the emulsion was thawed and centrifuged at 180 × g for 10 min. Furthermore, 10-fold serial dilutions of the
supernatant were prepared with brain heart infusion broth. The 50% lethal dose (LD50) was calculated by the method of
Kärber (8) after mice had been given 0.2 ml of the inoculum intraperitoneally.
Preparation of immune mice. BALB/c mice were immunized with 105 LD50 of each strain of R. tsutsugamushi by
subcutaneous injection. As soon as the mice showed symptoms of illness, a daily dose of 0.5 mg of tetracycline was
administered subcutaneously for 2 days.
Antigen preparation. All antigens were used as whole cell antigens. The Gilliam, Karp, and Kato strains of R. tsus-
tugamushi were grown in L cells. When the cytopathic effect was at a maximum, infected L cells were pelleted by
centrifugation at 18,000 × g for 20 min at 4°C, and the pellets were suspended in RPMI 1640 medium (GIBCO Labora-
tories, Grand Island, N.Y.). The rickettsiae were then liber-
ated from L cells with a Dounce homogenizer, centrifuged at
400 × g for 10 min to remove cell debris, and inactivated by
heat treatment at 56°C for 30 min. The cells were then
sonicated with an ultrasonic oscillator (UR 200P; Tomy,
Tokyo, Japan) at 20 kHz for 1 min. The samples were then
frozen at −80°C and preserved in this state. Antigen prepa-
ration was quantitated for total protein by the method of
Lowry et al. (16). In the evaluation of the antigenicity of
each rickettsial antigen, splenocytes obtained from immu-
nized mice were tested for lymphocyte proliferative re-

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sponse to the immunizing antigen. The optimal concentration of antigen was determined by examining lymphocyte proliferative responses to various antigen concentrations.

Establishment of a rickettsial antigen-specific T-cell line. (i) Preparation of splenocytes from immune mice and primary culture. Spleens were removed aseptically from BALB/c mice immunized 3 weeks previously, and single-cell suspensions were prepared by mincing and passing the cells through sterile stainless steel sieves (no. 200) into RPMI 1640 medium. The cells were then suspended in Tris-NH$_4$Cl, pH 7.2, to remove erythrocytes and washed three times with RPMI 1640 medium. Cells were adjusted to a concentration of 5 x 10$^6$ viable cells per ml in RPMI 1640 medium supplemented with 100 µg of streptomycin per ml–100 U of penicillin per ml–1% glutamine–5 x 10$^{-3}$ M 2-mercaptoethanol–10% heat-inactivated fetal calf serum (FCS; GIBCO). A 10-ml portion of the suspension was supplemented with Gillian antigen in a culture flask (Falcon no. 3013) so that the final definite concentration would be 50 µg/ml. The primary culture was performed at 37°C in a humidified 5% CO$_2$ atmosphere.

(ii) Preparation of filler cells. Splenocytes from normal BALB/c mice were used. Single-cell suspensions were prepared by the methods described above. After the cells had been washed three times, they were irradiated at 3,300 R and used as filler cells.

(iii) Long-term culture of the cell line. After 3 weeks in primary culture, the cells were harvested and viable cells were counted by the trypan blue exclusion test. The resulting viable cells and filler cells were mixed in RPMI 1640 medium supplemented as described above so that they would be adjusted to concentrations of 1 x 10$^6$ and 5 x 10$^6$ per ml, respectively, and a 10-ml portion was placed in each culture flask. Under these conditions, resting culture was performed for 14 days. The viable cells were then harvested and mixed with filler cells as described above, except that Gillian antigen was added so that the final concentration was 50 µg/ml. Under these conditions, stimulation culture was performed for 14 days. Stimulation culture and resting culture were then alternately repeated four times. Following this, cells capable of proliferation under these conditions were passaged every 14 days in the stimulation culture and then maintained in a long-term culture.

Cell surface phenotype. Viable cells on days 10 to 14 after passage were enriched by centrifugation over a ficoll-Conray gradient (specific gravity, 1.090; Immuno Biological Laboratories, Tokyo, Japan) at 400 x g for 30 min. The resulting cells were washed twice and adjusted to a concentration of 2 x 10$^7$ cells per ml in Dulbecco phosphate-buffered saline, pH 7.2, containing 1% FCS. Monoclonal anti-Thy-1.2, anti-L3T4, or anti-Lyt-2 (Becton Dickinson, Mountain View, Calif.) was added to samples of the cells, and the mixtures were incubated for 30 min on ice. After being washed three times, the cells were treated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins G, A, and M (Cooper Biomedical, Inc., West Chester, Pa.) for 40 min on ice. After the cells had been washed three times, 10$^4$ cells from each sample were analyzed for fluorescence on a log scale with an EPICS-C fluorescence-activated cell sorter (Coulter Electronics, Inc., Hialeah, Fla.). The control sample was treated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin alone.

Lymphocyte proliferation assay. The ability of the T-cell line to proliferate in response to rickettsial antigens was measured with a microculture assay system as follows.

Samples (50 µl) of filler cells containing 5 x 10$^5$ cells were placed in the wells of a 96-well microtiter U-bottom plate (Nunc, Roskilde, Denmark), and quadruplicate wells received either medium alone or dilutions of rickettsial antigens. After 60 min of culture, each well received a 100-µl sample of the T-cell line containing 5 x 10$^5$ cells, which were harvested on days 10 to 14 after passage and enriched in RPMI 1640 medium supplemented as described above. The cultures were incubated at 37°C in a 5% CO$_2$ atmosphere for 72 h. Tritiated thymidine (1 µCi; 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to each well for the final 12 h of culture, and incorporated radioactivity was determined after cells had been harvested onto glass filter strips by a multiple harvesting system. For evaluation of the antigenicity of rickettsial antigen, BALB/c mice were immunized with 10$^9$ LD$_{50}$ of each rickettsial antigen subcutaneously. At 5 weeks later, splenocytes from the mice were handled as described above, and into each microtiter well was placed a 100-µl sample containing 10$^5$ cells and one of various dilutions of rickettsial antigen. The data were expressed as net counts per minute, calculated by subtracting the number of counts per minute of cultures receiving only medium from the number of counts per minute of antigen-stimulated cultures, or as a stimulation index, calculated by dividing the number of counts per minute of antigen-stimulated cultures by the number of counts per minute of control cultures. Statistical analysis was carried out by using Student's t test.

Genetic restriction of antigenic response. To study the genetic restriction of the antigenic response, the T-cell line was tested for the response to Gilliam antigen with either syngeneic or allogeneic filler cells. Splenocytes from BALB/c (H-2$k$), NZB (H-2$^d$), and C57BL/6 (H-2$^b$) mice were used as filler cells.

Quantitation of interferon. The presence of interferon (IFN) in culture supernatants was detected by a modification of the 50% cytopathic effect method of Palmer et al. (24). Threefold dilutions of culture supernatants, which had been aseptically harvested 48 h after the start of the lymphocyte proliferation assay, were placed in triplicate wells of a 96-well microtiter plate (Falcon no. 3072) in 50-µl volumes. L cells were adjusted to a concentration of 4 x 10$^3$ viable cells per ml in Eagle minimum essential medium (Nissui, Tokyo, Japan) supplemented with 5% FCS. Cells were added to each well in 100-µl volumes and incubated for 12 h at 37°C in a humidified 5% CO$_2$ atmosphere. The samples were then removed, and the monolayers were washed twice with Eagle minimum essential medium without FCS and replaced with fresh medium containing vesicular stomatitis virus at a multiplicity which caused a complete cytopathic effect in unprotected cells within 48 h. At that time, cultures were observed for reduction of the cytopathic effect. The reciprocal of the highest dilution protecting 50% of the cells provided the IFN titer. Results were expressed in laboratory units.

Characterization of IFN. The IFN present in culture supernatants was characterized by acid stability and thermal stability. Samples were adjusted to pH 2.0 with 1 N HCl, allowed to stand at 4°C for 24 h, dialyzed against Eagle minimum essential medium, and sterilized by filtration. In another experiment, samples were treated in a 56°C water bath for 30 min or in an 80°C water bath for 10 min. These samples were then assayed for IFN activity.

Transfer of the rickettsial antigen-specific T-cell line. Each mouse was challenged with 10$^9$ LD$_{50}$ of R. tsutsugamushi Gilliam intraperitoneally. At 2 h later, the antigen-specific T
cells on days 10 to 14 after passage were enriched as described above and adjusted to 3.5 x 10^7 viable cells per ml in RPMI 1640 medium containing 10% FCS, and a 0.2-ml sample of the T cells was transferred to each mouse intraperitoneally. For the following 4 weeks, the conditions of the mice were observed. In the other groups, each mouse was injected with the same number of T cells (the population of Thy-1.2 antigen-positive cells being more than 95%), which had been purified from splenocytes of normal BALB/c mice by a modification of the nylon wool column method of Julius et al. (7), or given a rickettsial challenge alone.

RESULTS

Antigenicity of antigens and antigen dose response. In the lymphocyte proliferation assay, splenocytes obtained from mice immunized with the Gilliam, Karp, or Kato strain demonstrated responsiveness, with stimulation indices of 3.7, 4.4, and 3.6, respectively, to each homologous antigen. These data indicated that the antigenicities of the three antigens were nearly equivalent.

To determine the optimal antigen concentration in the proliferative response of the T-cell line, a wide range of antigen doses was used. The optimal proliferative response was elicited by using antigens diluted so as to contain 50 to 100 μg of protein per ml, and data from the lymphocyte proliferative response using 50 μg of protein per ml are presented.

Analysis of cell surface phenotype. To determine the expression of T-cell surface markers, the T-cell line was evaluated by flow cytometry with fluorescein isothiocyanate-conjugated monoclonal antibodies. The T-cell line stained brightly for Thy-1.2 (more than 97%), stained with the monoclonal antibody binding to the L3T4 antigen (more than 97%), and expressed no significant level of the Lyt-2 antigen (less than 1%). Histogram comparisons are shown in Fig. 1.

Antigen specificity of the T-cell line in the proliferative response. To determine the antigen specificity of the T-cell line, the line was tested for its reactivity to R. tsutsugamushi Gilliam, Karp, and Kato antigens and purified protein derivative antigen. The T-cell line reacted specifically (9,630 cpm) with Gilliam antigen, revealed partial cross-reactivity (4,150 cpm) with Karp antigen, and did not react with Kato and purified protein derivative antigens (Fig. 2). In the absence of either filler cells or antigen, proliferation was negligible. Furthermore, reactivity to antigens was maintained invariably in long-term cultures for more than six months. Thus, we concluded that the T-cell line was specific for R. tsutsugamushi Gilliam antigen.

Genetic restriction of the T-cell line. To determine whether the antigenic response of the T-cell line was restricted genetically, the line was tested for its antigen response with syngeneic and allogeneic filler cells. Splenocytes from BALB/c (H-2^d) or NZB (H-2^a) mice supported the proliferative response, whereas splenocytes from C57BL/6 (H-2^b) mice did not (Fig. 3). Thus, it was concluded that histocompatibility, probably within the H-2IA locus, between the T-cell line and filler cells was required for response of the cell line to rickettsial antigen.

IFN production in cultures of the T-cell line. Gilliam antigen induced high titers of IFN (729 U/ml) (Fig. 4). On the other hand, Karp and Kato antigens were capable of inducing low IFN titers (27 and 9 U/ml, respectively). We thus concluded that IFN production by the T-cell line was antigen specific.

Characterization of the IFN. The supernatants from cultures of the rickettsial antigen-specific T-cell line were characterized biochemically as indicated in Table 1. Heat treatment at 56°C for 30 min had no effect on the IFN titer. However, treatment at 80°C for 10 min destroyed the IFN activity completely. Treatment of the supernatants at pH 2.0...
for 24 h, followed by dialysis, also resulted in destruction of the IFN activity.

**Cell transfer.** Mice injected with the antigen-specific T-cell line all recovered and survived the rickettsial challenge (Table 2). However, mice given purified T cells derived from nonimmune mice or inoculated with rickettsiae alone all died 7 to 10 days after the rickettsial challenge.

**DISCUSSION**

Recently, remarkable progress has been made in culture techniques, and two methods for the long-term culture of normal T cells are now established. One is the method in which antigen-stimulated T cells are cultured with interleukin 2 (2), while the other involves the culture of T cells with antigen and the use of irradiated syngeneic splenocytes as filler cells (11, 31). In the present study, we established a continuous T-cell line specific for the Gilliam strain of *R. tsutsugamushi*, making use of the latter technical achievement. In its proliferative response, the T-cell line showed specific response to the Gilliam antigen and partial cross-reactivity with the Karp antigen. This observation coincides with the finding on lymphocyte proliferative response, obtained with immune-mouse splenocytes by Jerrells and Osterman (4), that mice immunized with Gilliam rickettsiae demonstrated cross-reactivity in response to Karp antigen. The cell line showed no response to Kato antigen, and it is not clear whether this was due to a difference in antigenicity or to the dropout of clones reactive to the antigen in response to the culture conditions.

It has been well documented that in major histocompatibility complex subregion I-A, identity between antigen-reactive T cells and filler cells is required for antigen-driven murine T-cell proliferative responses (11), and that T cells must recognize antigens in association with cell surface Ia antigens for them to be activated in this system. In the present study, the rickettsial antigen-specific T-cell line also revealed genetic restriction in its proliferative response.

The data presented in this study clearly show that the rickettsial antigen-specific T-cell line produces IFN after antigen stimulation. Furthermore, because the IFN-γ was produced from T cells and the activity was unstable at pH 2.0, IFN derived from the cell line might be considered to belong to the IFN-γ subclass (28). The importance of the T-cell–macrophage system in protection against rickettsial infection has been supported by studies on aspects such as (i) transfer of sensitized T lymphocytes, (ii) lymphokine production, (iii) activation of macrophages by lymphokines, and (iv) delayed-type hypersensitivity to rickettsial antigens (23). Rickettsicidal activity has been found in activated macrophages obtained after lymphokine treatment in vitro and in peritoneal macrophages obtained after intraperitoneal rickettsial inoculation, and tumoricidal activity has also been found in these macrophages. Some data indicate that these macrophages are activated nonspecifically by lymphokines (19, 20, 21). It has also been reported that IFN-γ plays an important role in the T-cell–macrophage system, since the IFN-γ titer in serum and production of IFN-γ by T lymphocytes in vitro both increase in parallel with the acquisition of resistance against rickettsial infection (25), and that the lymphokine which activates rickettsicidal activity in macrophages is IFN-γ (6), one of the macrophage-activating factors (22). On the other hand, destruction of rickettsiae by macrophages seems to be insufficient for eliminating rickettsiae in the host, since endothelial cells (nonphagocytic phagocytes) are also major sites of rickettsial multiplication. Therefore, it has been supposed that destruction of infected cells by sensitized T cells or establishment of conditions unsuitable for rickettsial survival and growth in nonprofessional phagocytes would constitute an important mechanism of host defense against rickettsial infection (32). Recently, it has been reported that IFN-γ inhibits rickettsial growth in nonphagocytic cells (32, 33) and that it has a specific cytolytic action on cells infected with rickettsiae (35), showing various antirickettsial activities in systems without macrophages. We are of the opinion that it is particularly important in protection against infection for IFN-γ to be produced antigen specifically. It is considered that IFN-γ produced from the T-cell line in this way would act on infected cells to inhibit rickettsial growth directly or through activation of macrophages in the host.

Studies on IFN-γ production in alloantigen (12)- or viral antigen (18)-specific and other types of T cells have proved that both cytotoxic and helper T cells are able to produce IFN-γ (15). In the present study, the rickettsial antigen-specific T-cell line stained brightly for L3T4 antigen, which is expressed by H-2 complex class II antigen-restricted T

**TABLE 1. Characterization of IFN**

<table>
<thead>
<tr>
<th>Treatment of supernatant</th>
<th>IFN titer (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>729</td>
</tr>
<tr>
<td>56°C, 30 min</td>
<td>729</td>
</tr>
<tr>
<td>80°C, 10 min</td>
<td>&lt;5</td>
</tr>
<tr>
<td>pH 2.0, 24 h</td>
<td>5</td>
</tr>
</tbody>
</table>

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* Characterization of IFN produced in cultures of the Gilliam antigen-specific T-cell line. Samples were treated as described in the text.

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**TABLE 2. Protective effect by cell transfer**

<table>
<thead>
<tr>
<th>Cell transfer</th>
<th>No. of survivors/total</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen-specific T cells</td>
<td>5/5</td>
<td>100</td>
</tr>
<tr>
<td>Nonimmunized T cells</td>
<td>0/5</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0/5</td>
<td>0</td>
</tr>
</tbody>
</table>

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* Protective effect against rickettsial challenge by the Gilliam antigen-specific T-cell line. Syngeneic recipient mice were infected with 10^5 LD_{50} of the Gilliam strain before inoculation of antigen-specific cell transfer. Control mice received either purified T cells derived from nonimmunized mice or a rickettsial challenge alone.

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**FIG. 4. IFN production in cultures of the Gilliam antigen-specific T-cell line. The T-cell line was cocultured with syngeneic filler cells and rickettsial antigen for 48 h. Supernatants were tested for IFN activity. Results are expressed in laboratory units for triplicate cultures.**
cells, i.e., helper T cells (17, 34). Therefore, it was shown that production of IFN-γ in rickettsial infection is, at least partially, carried out by helper T cells.

In the present study, an obvious protective effect was observed through transfer of the rickettsial antigen-specific T-cell line into mice. This indicated that antigen-specific helper T cells, which receive a signal to proliferate from antigen-presenting cells, play an important role in the protective system against rickettsial infection. As for the mechanism, it is supposed that IFN-γ produced antigen specifically by the T-cell line acts in a rickettsicidal manner either directly or through macrophages in vivo. In addition, the presence of lymphoheme-activated killer cells (1, 27) and rickettsial antigens on the surface of infected cells (26) has been reported recently. It is also supposed that lymphoheme-activated killer cells, induced by interleukin 2 produced by transferred T cells, has an injurious action on infected cells, since helper T cells are able to produce interleukin 2 by antigenic stimulation (30).

A T-cell line which can be maintained in long-term culture has thus been shown to be a valuable tool for investigating T-cell response to antigens, especially in diseases for which cell-mediated immunity and lymphokines are known to be of primary importance.

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