ANTIGEN-SPECIFIC PRODUCTION OF IMMUNE INTERFERON
BY T CELL LINES*

By J. L. McKimm-Breschkin, P. L. Mottram, W. R. Thomas and
J. F. A. P. Miller

From The Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital,
Victoria 3050, Australia

The production of immune γ-interferon (IFN-γ) after mitogen stimulation of T cell
populations is now well established (1, 2). Antigen-specific IFN-γ induction has only
been demonstrated in sensitized animals, or in vitro by lymphocytes obtained from
these animals after challenge with specific antigen (3–6). Sonnenfeld et al. (6), using
a panel of serum to define the cell population responsible for antigen-specific IFN-γ
induction, suggested that cytotoxic T lymphocytes in cooperation with B lymphocytes,
macrophages, or both, produced IFN-γ. It was found in subsequent work with anti-T
cell monoclonal reagents that OK-T8 antibodies that recognized the cytotoxic/suppressor cells failed to induce IFN-γ (7). However, marginal levels of IFN-γ were
obtained with OK-T4 antibodies that recognized the helper subpopulation. The
cellular interactions required for IFN-γ production are thus apparently as complex as
other immune responses and could be more easily studied with T cell lines.

We investigated the ability of antigen to induce IFN-γ in two continuous T cell
lines that have been shown to mediate hapten-specific delayed hypersensitivity (DTH)
to the contact sensitizing agent, oxazolone. Both cell lines produced IFN-γ after
stimulation by specific antigen. The IFN-γ induction was restricted by the I region of
the MHC. The simplicity and reproducibility of this assay system will provide us
with considerable potential for testing T lymphocyte function at the clonal level.

Materials and Methods

Mice. 6–8-wk-old BALB/c, CBA, (CBA × BALB/c)F1, BALB/c (H-2^k), and A.TL mice
were obtained from The Walter and Eliza Hall Institute of Medical Research.

Preparation of Antigen-presenting Cells (APC). The 4-ethoxymethylene-2-phenyl oxazolone (oxazolone) and picryl chloride (BDH Chemicals, Ltd., Poole, United Kingdom) were dissolved in ethanol at concentrations of 3% and 5%, respectively. A total of 100 µl of antigen solution was
painted on the clipped thorax, abdomen, and forepaws of mice. The following day a cell
suspension was prepared from the inguinal, subcapsular, and axillary lymph nodes, in Hepes-
 BUFFERED EAGLE'S MEDIUM (Flow Laboratories, Stanmore, Australia). Cells were washed and irradiated (2,000 rad at 900 rad/min, Phillips RT250 x-ray machine). Cells were washed again and added to the lymphocyte cultures.

Cells and Culture Media. Lymphocytes were cultured in RPMI 1640 (Flow Laboratories)
containing 5 × 10^{-3} M 2-mercaptoethanol (Calbiochem-Behring Corp; American Hoechst

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1 Thomas, W. R., P. L. Mottram, and J. F. A. P. Miller. Hapten-specific T cell lines mediating delayed
hypersensitivity to contact sensitizing agents. Manuscript submitted for publication.
Corp., La Jolla, CA), 2 mM L-glutamine (Commonwealth Serum Laboratories, Parkville, Australia), and 10% heat-inactivated fetal calf serum (Flow Laboratories). For long-term cultures, this was supplemented with supernatant from concanavalin A-stimulated spleen cell cultures (CAS) as described in detail by Thomas et al.1

The lymphocyte cultures were maintained as described. Briefly, 10⁶ antigen reactive cells, 10⁶ irradiated syngeneic spleen filler cells, and 10⁵ irradiated APC in 1.5 ml of medium supplemented with CAS were added to each well of Costar 24-well cluster trays (Costar 3524, Costar, Data Packaging, Cambridge, MA). Cells were recultured weekly after separation on Ficoll paque gradients (density 1.077; Pharmacia, Upsala, Sweden). Lymphocytes for interferon assays were washed, separated on Ficoll paque gradients, washed again, and resuspended in medium as described above without CAS.

The L cells used for interferon assays were originally obtained from Dr. M.-L. Lohmann-Matthes (Max Planck Institut für Immunobiologie, Freiburg) and were cultured in RPMI 1640 containing 7.5% heat-inactivated fetal calf serum. The overlay medium used for plaque assay was normal Eagle's medium diluted 1:1 with 1% agarose (Miles Laboratories, South Africa).

Interferon Induction and Assay. Lymphocytes were distributed at 10⁶ cells/well in 1.5 ml of medium in Costar 24-well dishes. Irradiated APC were added to each well at the required concentration, and the cultures were incubated at 37°C in a humidified atmosphere of 10% CO₂. Culture supernatants were usually harvested 48 h after the initial stimulation.

Twofold dilutions of the supernatant fluids were assayed for interferon by the plaque-reduction method, essentially as described previously (8). 3 × 10⁵ mouse L cells were used for the assay, and Semliki Forest virus, which had been grown in L cells, was used as the indicator virus. Titers are expressed as the reciprocal of the dilution causing a 50% reduction in the number of plaques compared to the control plates (PRD₅₀). An internal standard was included in each assay. All samples from each experiment were assayed simultaneously.

Results

The challenge of sensitized lymphocytes with specific antigen may invoke the production of several lymphokines, including γ interferon (immune, type II), (3–5). We have identified a product demonstrating antiviral activity from the culture supernatants of oxazolone-specific continuous T cell lines. The antiviral activity was characterized as IFN-γ because it was species specific, stable at 56°C for 30 min, and totally inactivated by treatment at pH 2 and by treatment with 100 µg/ml of trypsin (3, 6).

Antigen Specificity and Dose-Response of IFN-γ Induction. The titers of interferon produced when the 010 (CBA anti-oxazolone) and 05 (BALB/c anti-oxazolone) cell lines were stimulated with different numbers of APC are shown in Tables I and II, respectively. Both cell lines produced higher titers of IFN-γ when stimulated with increasing numbers of oxazolone-specific APC. A minimum of 2 × 10⁴ APC was required for induction of detectable IFN-γ. Optimal induction occurred when the ratio of APC to oxazolone-specific lymphocytes was >10:1. Oxazolone-specific induction of IFN-γ was demonstrated for both lines, because neither normal lymph node cells nor cells presenting an unrelated antigen, e.g., picryl chloride, could induce detectable levels of IFN-γ. A decrease in interferon yield was also observed when the dose response experiment was repeated with the addition of irrelevant APC to keep the total number of APC constant (Table I, experiment 2).

MHC Restriction of IFN-γ Induction. We used several inbred strains of mice to determine whether antigen presentation was MHC-restricted (Table III). There was no response by either line to allogeneic APC. When the 010 line (CBA, H-2k) was stimulated with congenic BALB/c H-2k cells, induction occurred, demonstrating that the oxazolone response was H-2k restricted. Further, because induction occurred with
TABLE I
Antigen Specificity of Interferon Induction by the 010 Line
(CBA-Anti-Oxazolone)

<table>
<thead>
<tr>
<th>Number of 010 cells</th>
<th>Syngeneic APC</th>
<th>Number IFN titer (PRDso)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^5$ Oxazolone</td>
<td>$10^6$</td>
<td>200</td>
</tr>
<tr>
<td>$10^4$ Oxazolone</td>
<td>$3 \times 10^5$</td>
<td>100</td>
</tr>
<tr>
<td>$10^3$ Oxazolone</td>
<td>$10^5$</td>
<td>20</td>
</tr>
<tr>
<td>$10^2$ Oxazolone</td>
<td>$3 \times 10^4$</td>
<td>5</td>
</tr>
<tr>
<td>$10^1$ Picryl chloride</td>
<td>$10^4$</td>
<td>&lt;5</td>
</tr>
<tr>
<td>$10^0$ Normal</td>
<td>$5 \times 10^3$</td>
<td>&lt;5</td>
</tr>
<tr>
<td>0</td>
<td>$5 \times 10^2$</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$10^4$ Oxazolone:picryl chloride</td>
<td>5 x 10^5:0</td>
<td>30</td>
</tr>
<tr>
<td>$10^3$ Oxazolone:picryl chloride</td>
<td>2 x 10^5:3 x 10^5</td>
<td>20</td>
</tr>
<tr>
<td>$10^2$ Oxazolone:picryl chloride</td>
<td>1 x 10^5:4 x 10^5</td>
<td>5</td>
</tr>
<tr>
<td>$10^1$ Oxazolone:picryl chloride</td>
<td>0.5 x 10^5:4.5 x 10^5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

TABLE II
Antigen Specificity of Interferon Induction by the 05 Line
(BALB/c Anti-Oxazolone)

<table>
<thead>
<tr>
<th>Number of 05 cells</th>
<th>Syngeneic APC</th>
<th>Number IFN titer (PRDso)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^5$ Oxazolone</td>
<td>$1 \times 10^6$</td>
<td>50</td>
</tr>
<tr>
<td>$10^4$ Oxazolone</td>
<td>$3 \times 10^5$</td>
<td>20</td>
</tr>
<tr>
<td>$10^3$ Oxazolone</td>
<td>$2.5 \times 10^4$</td>
<td>10</td>
</tr>
<tr>
<td>$10^2$ Oxazolone</td>
<td>$1.25 \times 10^4$</td>
<td>&lt;5</td>
</tr>
<tr>
<td>$10^1$ Picryl chloride</td>
<td>$1 \times 10^4$</td>
<td>&lt;5</td>
</tr>
<tr>
<td>$10^0$ Normal</td>
<td>$1 \times 10^3$</td>
<td>&lt;5</td>
</tr>
<tr>
<td>0</td>
<td>$1 \times 10^2$</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

TABLE III
MHC Restriction of Interferon Induction by 05 and 010 Lines

<table>
<thead>
<tr>
<th>Source of APC</th>
<th>IFN titer (PRDso)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA</td>
<td>&lt;5</td>
</tr>
<tr>
<td>BALB/c</td>
<td>20</td>
</tr>
<tr>
<td>BALB/c-H-2d</td>
<td>&lt;5</td>
</tr>
<tr>
<td>CBA x BALB/c</td>
<td>50</td>
</tr>
<tr>
<td>A.TL</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

$10^5$ antigen reactive cells were stimulated by $3 \times 10^5$ APC. Supernatants were harvested after 48 h for IFN assay.

A.TL APC which are only matched at the major histocompatibility complex (MHC) I region, the response to oxazolone must be I region restricted. A similar restriction was shown for the 05 BALB/c H-2d, where induction only occurred with syngeneic and (CBA x BALB/c)F1 APC.

Kinetics of IFN-γ Production. The kinetics of IFN-γ production by the 010 line are shown in Fig. 1. Culture supernatants were harvested at the indicated time intervals and assayed for IFN activity. IFN-γ was first detected 6–12 h after stimulation and reached maximum levels by 30 h. Once this level was reached the IFN titer remained constant for at least another 3 d.
To determine whether the IFN-γ was stable, or whether it was continually being degraded and newly synthesized, IFN-γ production was measured between time points. Supernatants from several cultures were removed every 6 h and replaced with fresh medium. IFN titers were then determined for consecutive time intervals (Fig. 1). Results showed that even in the presence of continued antigenic stimulation, no further IFN-γ was produced after 36 h of stimulation. The IFN-γ released before this time was stable to incubation at 37°C.

Discussion

Mitogen stimulation of IFN-γ production by T cell lines (2) and T cell clones (1) has recently been reported. We were therefore interested in investigating IFN-γ production by T cell lines and clones after antigenic stimulation. We have two oxazolone-specific T cell lines that have been passaged in tissue culture for at least 6 mo.1

After stimulation with specific APC, yields of IFN-γ of 200 U/ml (or 300 U/culture well) were obtained from as few as 10⁵ oxazolone-specific T lymphocytes. The levels of IFN-γ produced were dependent upon the amount of antigenic stimulation. A minimum of 2 × 10⁴ oxazolone APC was required for stimulation of detectable IFN-γ production. Optimal induction of IFN-γ was achieved by increasing the numbers of APC to 10⁶. The T cell lines did not produce IFN-γ when normal lymph node cells, or lymph node cells presenting the unrelated antigen picryl chloride, were added to the cultures. The IFN was not produced by the APC in response to any factor released from the T cells. When irrelevant APC were added to maintain constant APC numbers, the titer of IFN again decreased in response to decreasing oxazolone APC. Confirmation of the cellular origin of the IFN was obtained by treatment of the cells for 2 h with 0.5 μg/ml of emetine hydrochloride, an inhibitor of protein synthesis. The drug inhibited production of IFN only when the T lymphocytes were treated (data not shown).

Kinetics of IFN-γ production demonstrated that maximal titers were obtained by 1-2 d after initial stimulation. Once produced, this IFN-γ was stable at 37°C in
cultures for at least a further 3 d. No further de novo synthesis of IFN-γ occurred after the first 36 h. These kinetics differed from those reported for mitogen stimulated IFN-γ production, where maximal titers were reached 3-4 d after stimulation (2, 7). Our kinetics more closely resembled those shown for induction of IFN-γ by monoclonal antibodies recognizing T cell antigens (7).

IFN is known to be a potent regulator of the immune system (9) including DTH (10). The timing of IFN administration relative to antigen is crucial. Administration before antigen can decrease or totally inhibit the sensitization. IFN given at the same time or after antigen has no effect or even enhances DTH (10). Because IFN-γ was not produced by the lines until 6–12 h after antigenic stimulation in vitro, IFN production in vivo would coincide with onset of DTH and may even enhance the response.

IFN-γ production was MHC restricted, and the response was controlled at least by the I region. DTH to protein antigens has been shown to be I region restricted (11-13). There is also a strong correlation between the Lyt-1 helper/DTH subset of T cells (12, 13) and I region restriction, as opposed to K,D restriction of the Lyt-1-2+ cytotoxic/suppressor subset. Because these oxazolone-specific T cell lines produced an I-restricted DTH in vivo and IFN-γ induction in vitro was I region restricted, these may be functions of the same cell. Preliminary work with clones from these T cell lines supports this. Some clones demonstrated antigen-specific induction of IFN-γ. Screening of clones by this assay also correlated extremely well with the results from DTH assays in vivo. Clones that lost the ability to produce IFN-γ in response to antigenic stimulation also failed to elicit DTH in vivo.

We have observed similar antigen-specific induction of IFN-γ, not only in the oxazolone system, but also with a T cell line reactive to the hapten azobenzene arsonate. Thus antigen specific induction of IFN-γ by continuous T cell lines provides a useful system for studying not only IFN-γ production, but for evaluating the response to particular antigens.

Summary

Continuous cultures of T cells reactive to the hapten 4-ethoxymethylene-2-phenyl-oxazolone were tested for interferon production after antigenic stimulation in vitro. Induction of interferon was antigen-specific and also restricted by the I region of the major histocompatibility complex. Kinetics of antigen induced interferon production were different from those reported for mitogen induced synthesis.

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References