INFECTION AND IMMUNITY, May 1980, p. 542-545
0019-9567/80/05-0542/04$02.00/0

Vol. 28, No. 2

Enumeration of Immune Interferon-Producing Cells Induced by Allogeneic Stimulation

YASUHIKO ITO,[1]* HIIZU AOKI,[1] YOSHINOBU KIMURA,[1] MICHIKO TAKANO,[1] KOICHIRO MAENO,[1] AND KAORU SHIMOKATA[2]

Germfree Life Research Institute[1] and First Department of Internal Medicine,[2] Nagoya University School of Medicine, Showa-Ku, Nagoya, Japan

We succeeded in enumerating immune interferon-producing cells induced by allogeneic stimulation, and proved that they were indeed T lymphocytes. The peak level of these cells in spleen was attained on day 5 after immunization, was maintained for about 2 days, and declined thereafter. Titers of interferon produced in vitro by sensitized spleen cells were maximum on day 7. This suggests that the maturation of immune interferon-producing cells follows cell proliferation after antigen stimulation.

Downloaded from http://iai.asm.org/ on November 25, 2020 by guest

Thymus-derived lymphocytes (T cells) play a central role in cell-mediated immunity. At present, however, the number of specifically sensitized T cells and the kinetics of these cells are poorly understood. Enormous progress in understanding the mechanisms of antibody formation was made as a direct consequence of Jerne's plaque assay technique for antibody-producing cells. All of the methods of assaying T cell-mediated function, on the other hand, except for the virus plaque assay method developed by Bloom et al. (1), can only measure the biological activities of a certain cell population, and do not enumerate antigen-specific T cells at a single cell level, analogous to Jerne's method. Antigen-sensitive T cells are able to produce a variety of soluble factors, such as macrophage migration inhibitory factor, lymphotoxin, and immune interferon. Osborn and Walker (2) introduced a method for the detection of interferon production by individual spleen cells of mice after intravenous virus infection. This paper presents a new method for enumerating immune interferon-producing cells (IIPC) in the spleens of mice sensitized by allogeneic stimulation, and describes studies of the evolution of the immune interferon-producing cell response in the spleens of antigen-stimulated mice.

MATERIALS AND METHODS

Mice. Male C57BL/6 mice weighing 25 to 30 g were used throughout the study.

Cell culture. Mouse L cells (derived from C3H mice) were grown in Eagle minimal essential medium (MEM) supplemented with 10% bovine serum, 10% tryptose phosphate broth, and antibiotics. Culture medium for mouse spleen cells was MEM supplemented with 5% fetal calf serum.

Interferon assay. Interferon was assayed by the cytopathic effect inhibition microassay method previously described (3), using L cells and vesicular stomatitis virus as challenge virus. One interferon unit in our laboratory was equivalent to 2.7 international reference units of mouse interferon.

Antiserum. Anti-mouse immunoglobulin serum was obtained from Fujizoki Pharmaceutical Co., Japan. Anti-H-2^b and anti-I region-associated antigen (8, 9) antiserum were purchased from Searle Diagnostic Co., England. Anti-thy1-2 antiserum was prepared according to the method of Takahashi et al. (4).

RESULTS

Enumeration of IIPC. Enumeration of IIPC was performed in the following manner. Whole spleens were aseptically removed from mice preinjected intraperitoneally with 10^7 L cells, and were teased on a steel mesh immersed in chilled Eagle in a plastic dish. The cells that passed through were washed twice with medium, and L-cell-sensitized spleen cells were cocultivated with L-cell monolayers in plastic dishes (Falcon 3002). Almost all of the sensitized spleen cells adhered specifically to antigenic cells (L cells) within 1 to 2 h (unpublished data). Three hours after cocultivation, monolayers of L cells were washed, and 2.5 ml of MEM, containing 0.3% agar, 5% fetal calf serum, and bicarbonate, was poured onto these monolayers and allowed to gel in a cold room for 5 min. L cells were incubated at 35°C for another 12 h, and then the agar was removed. The cells were washed with MEM to remove all traces of agar, and inoculated with about one plaque-forming unit of vesicular stomatitis virus per cell. After virus was adsorbed at 35°C for 1 h, MEM was added, and the monolayers were incubated at 35°C for 2 to 3 days until control monolayers were completely lysed. After vigorous washing to remove dead cells, the remaining cells were stained with May-Gruenwald-Giemsa solution.

Foci of protected L cells are shown in Fig. 1A, and one focus observed by microscope is pre-

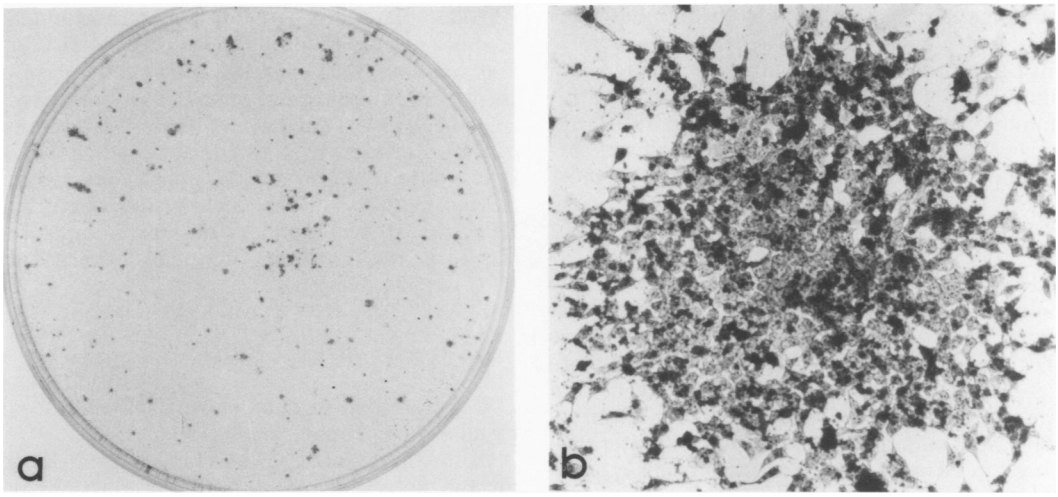

FIG. 1. *Foci produced on an L-cell monolayer. Mouse spleen cells were derived from C57BL/6 mice injected with 10^7 L cells 7 days previously. (a) Whole petri dish; (b) one focus. Magnification, ×110.*

sented in Fig. 1B. Counting of foci was checked by microscopic observation.

Since cycloheximide was found to inhibit either the production or the action of interferon, we examined the effect of cycloheximide on formation of protected foci of L cells. Three hours after cocultivation between L cell-sensitized spleen cells and L cells, monolayers of L cells were washed, and soft agar containing 10 μg of cycloheximide per ml was poured onto these monolayers. Twelve hours later, the agar was removed, and cell monolayers were washed three times with MEM and inoculated with vesicular stomatitis virus. Inhibition of protein synthesis completely blocked formation of protected foci (Table 1). In addition, no foci were observed when L cell-sensitized spleen cells were overlaid on monolayers of FL cells, whose origin is human amnion (unpublished data).

A consistent linear relationship between number of spleen cells and and number of foci in a dilutional series suggested that a single spleen cell accounted for a given protected focus (Fig. 2).

Cell surface antigens of IIPC. In a preceding publication (1a), we showed that treatment of L cell-sensitized spleen cells with anti-thy1-2 antiserum plus rabbit serum before cocultivation with L cells completely abolished the immune interferon production. The following experiments were performed to investigate whether or not the focus-forming cells were indeed T cells. Spleen cells from mice primed 7 days previously with 10^7 L cells were cocultivated with L-cell monolayers for 3 h. Monolayers of L cells were washed and then were treated with anti-thy1-2 antiserum plus complement at 37°C. Thirty minutes after treatment, assay for IIPC was performed. Treatment with anti-thy1-2 antise-

TABLE 1. *Effect of cycloheximide treatment on focus formation*[a]

Treatment	No. of foci[b] per 10^5 cells
MEM	463 ± 49
Cycloheximide	5 ± 2

[a] Spleen cells from C57BL/6 mice primed 7 days previously with 10^7 L cells were cocultivated with L-cell monolayers for 3 h. Monolayers of L cells were washed, and soft agar containing 10 μg of cycloheximide per ml was poured onto these monolayers. Twelve hours later, the agar was removed, and cells were washed three times with MEM and inoculated with vesicular stomatitis virus.

[b] Mean ± standard deviation of the mean.

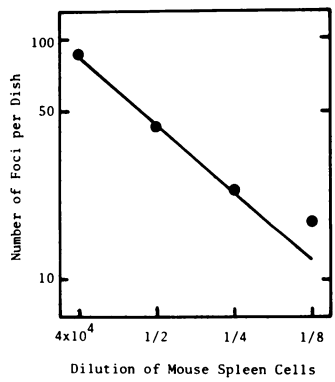

FIG. 2. *Effects of L cell-sensitized mouse spleen cell numbers upon foci formation. Serial twofold dilutions of spleen cells were cocultured with L-cell monolayers.*

Downloaded from http://iai.asm.org/ on November 25, 2020 by guest

rum plus complement completely eliminated focus counts (Table 2). Complement alone or anti-thy1-2 antiserum alone scarcely affected the number of foci, and treatment with anti-immunoglobulin antiserum or anti-Ia antiserum plus complement showed no effect on focus formation (Table 3), strongly suggesting that most of the IIPC belonged to the T-cell population. H-2 antigen(s) was expressed on the surface of IIPC (Table 3).

Evolution of IIPC in the spleen. Next, the following experiments were undertaken to investigate the development of T cells detected as IIPC after immunization of L cells. Mice were injected intraperitoneally with 10^7 L cells and were killed at various times after immunization. IIPC were enumerated, and interferon titer produced in vitro by sensitized spleen cells cocultivated with L cells was measured simultaneously (Fig. 3A). The peak level of IIPC was attained on day 5 after immunization, was maintained for about 2 days, and declined thereafter. In contrast, titers of interferon produced by sensitized spleen cells were maximum on day 7. The ratio of the interferon titer to the number of IIPC, that is, interferon-producing capacity per cell, is plotted in Fig. 3B. This finding indicated that IIPC increased in number until day 5, and subsequently the interferon-producing capacity of IIPC was augmented.

TABLE 2. *Effect of anti-thy1-2 antiserum treatment of focus formation*[a]

Treatment at 37°C	No. of foci[b]
MEM	54.5 ± 1.5
Complement[c]	52.5 ± 5.5
Anti-thy1-2 antiserum[d]	51.0 ± 7.0
Anti-thy1-2 antiserum + complement	2.9 ± 2.9

[a] 2.5×10^4 spleen cells from C57BL/6 mice primed 7 days previously with 10^7 L cells were cocultivated with L-cell monolayers for 3 h. Monolayers of L cells were washed and then were treated with anti-thy1-2 antiserum plus complement. Thirty minutes after treatment, the monolayers were overlaid with agar.
[b] Mean ± standard deviation of the mean.
[c] Absorbed rabbit serum, 1:16.
[d] Anti-thy1-2 AKR mouse serum (1:10).

TABLE 3. *Effects of treatment of various antisera on focus formation*[a]

Expt no.	Antiserum (dilution)	No. of foci[b] −Complement	No. of foci[b] +Complement
1	MEM	225 ± 16	212 ± 23
	Anti-H-2^b antiserum[c] (×300)	222 ± 7	65 ± 2
	Anti-Ia antiserum[d] (×4)	235 ± 1	210 ± 2
	Anti-mouse immunoglobulin antiserum (×10)	240 ± 20	222 ± 15
1	MEM		152
	Anti-H-2^b antiserum (×10)		<1
	(×30)		<1
	(×90)		18 ± 10
	(×270)		51 ± 15

[a] Spleen cells (10^5 in experiment 1, 3.3×10^4 in experiment 2) from C57BL/6 mice primed 7 days previously with 10^7 L cells were cocultivated with L-cell monolayers for 3 h. Monolayers of L cells were washed and then were treated with various reagents. Thirty minutes after treatment, the monolayers were overlaid with agar.
[b] Mean ± standard deviation of the mean.
[c] B10Br anti-C57BL/10ScSn serum.
[d] B10A anti-C57BL/10ScSn serum absorbed with EL4 cells.

DISCUSSION

We succeeded in enumerating IIPC induced by allogeneic stimulation.

Osborn and Walker (2) had reported a method for the detection of interferon production by individual spleen cells of mice after intravenous virus infection. However, we found it difficult to obtain reproducible results with their methods. When, according to their methods, interferon-producing cell-agar mixture was poured onto a confluent monolayer of target cells and allowed to gel at room temperature, IIPC were not fixed firmly on the target cells. The cells for titration of interferon were identical with antigenic cells in our system. Since almost all of IIPC induced by allogeneic stimulation adhered specifically to antigenic cells within 1 to 2 h (unpublished data), IIPC were cocultured with antigenic L cells in liquid culture for the first 3 h. Subsequently, after monolayers of antigenic L cells were washed, soft agar was poured onto these monolayers and allowed to gel in a cold room for 5 min. By using these methods, we were able to obtain reproducible results.

When L cell-sensitized spleen cells of C57BL/6 mice were treated with anti-thy1-2 antiserum or anti-H-2^b antiserum plus complement after cocultivation with L-cell monolayers for 3 h, i.e., just before being overlaid with agar, there was about a 95% decrease in the number of IIPC. On the other hand, treatment with anti-immunoglobulin antiserum or anti-Ia antiserum plus complement showed no effect on IIPC. These findings strongly suggest that most of the IIPC belong to the T-cell population. In our previous study (1a), treatment of L cell-sensitized spleen cells of C57BL/6 mice with anti-thy1-2 antiserum plus complement before cocultivation with L cells completely abolished immune interferon production. However, this method for the assay of interferon activity in culture fluids could only certify that T cells participated in immune interferon production. Our enumeration method can clarify directly the cell surface antigen(s) of IIPC.

Downloaded from http://iai.asm.org/ on November 25, 2020 by guest

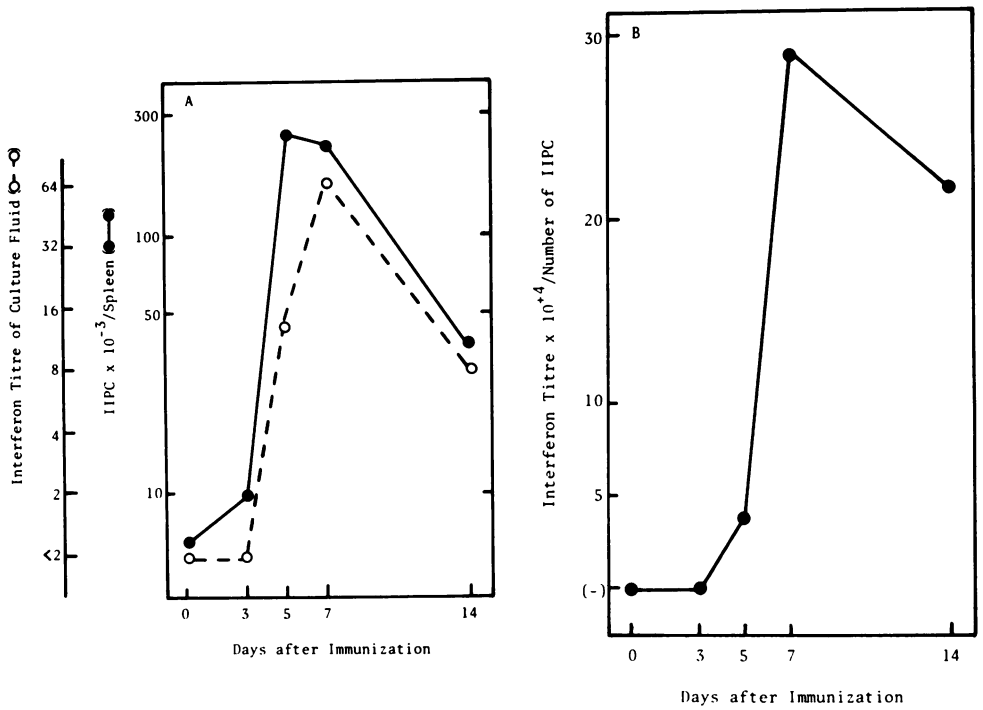

FIG. 3. *Kinetic study of IIPC and immune interferon production. (A) Mice were injected intraperitoneally with 10^7 L cells and killed on various days after immunization. Mouse spleen cells were incubated in MEM supplemented with 5% fetal calf serum at 35°C. IIPC were enumerated, and interferon titer produced in vitro by sensitized spleen cells (one-tenth spleen cells of whole spleen) that had been cocultivated for 24 h with L cells was measured.* (●) *IIPC.* (○) *Immune interferon titer. (B) The kinetics of the ratio of the interferon titer to the number of IIPC.*

IIPC increased in number until day 5, and then the interferon-producing capacity of IIPC was augmented. The high level of interferon-producing capacity per cell seemed to be sustained for rather a long time after immunization. This indicates that the maturation of T cells follows cell proliferation after antigenic stimulation.

As far as we know, this is the first report concerning the enumeration of lymphokine-producing T cells. The establishment of methods for enumerating lymphokine-producing T cells would make it possible to analyze the nature of effector cells and cell-to-cell cooperation in cell-mediated immunity.

LITERATURE CITED

1. **Bloom, B. R., L. Jimenez, and P. L. Marcus.** 1970. A plaque assay for enumerating antigen-sensitive cells in delayed hypersensitivity. J. Exp. Med. **132**:16-30.
1a. **Ito, Y., Y. Nishiyama, K. Shimokata, H. Takeyama, and A. Kumi.** 1979. Immune interferon produced in vitro as a quantitative indicator of cell-mediated immunity. Microbiol. Immunol. **23**:1109-1177.
2. **Osborn, J. E., and D. L. Walker.** 1969. The role of individual spleen cells in the interferon response of the intact mouse. J. Exp. Med. **62**:1038-1045.
3. **Shimokata, K.** 1978. Studies on the pathogenicity of human-origin parainfluenza virus in the brain of mice. Microbiol. Immunol. **22**:535-543.
4. **Takahashi, T., L. J. Old, and E. A. Boyse.** 1970. Surface alloantigens of plasma cells. J. Exp. Med. **131**:1325-1341.

Downloaded from http://iai.asm.org/ on November 25, 2020 by guest