INFECTION AND IMMUNITY, Feb. 1981, p. 519-523
0019-9567/81/020519-05$02.00/0

Vol. 31, No. 2

Natural Interferon-Producing Cells in Mice

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When mouse lymphoid cells derived from untreated C57BL/6 mice were cocultivated in liquid cultures with L-cell monolayers for 3 h, overlaid with soft agar, and then further incubated for 12 h, protected foci of L cells against vesicular stomatitis virus infection were formed. Many strains of mice have been found to have the protected focus-forming cells on the L-cell monolayers. The formation of the protected foci was completely suppressed by addition of cycloheximide into soft agar. When anti-interferon (type I) antiserum was added to soft agar, there was a decrease in focus counts of about 80%. These experimental results indicated that the formation of protected foci by untreated mouse lymphoid cells was mediated for the most part by type I interferon that was produced without addition of special inducer. We have designated these focus-forming cells as natural interferon-producing cells (NIPC). NIPC belong to the glass-adherent fraction, and Thy-1 antigen, immunoglobulin, and Ia antigen could not be detected on the surface of the NIPC. NIPC were detected in congenitally athymic nude mice. These findings suggest that NIPC belong to the Ia-negative macrophages. Mouse lymphoid cells obtained from germfree mice could form the protected foci on L-cell monolayers and could produce interferon without the addition of a special inducer. NIPC are considered to be a cellular background for spontaneous interferon production.

We have recently succeeded in enumerating immune interferon-producing cells (IIPC) induced by allogeneic stimulation (2). In this system, L-cell-sensitized spleen cells adhered specifically to the antigenic L-cell monolayers and could produce immune interferon (2). Consequently, L cells surrounding IIPC, when overlaid with soft agar, acquired the resistance against virus infection. Therefore, even when a large amount of vesicular stomatitis virus (VSV) was inoculated into mixed-cell cultures, foci of protected cells remained. In the course of this investigation, we found that nonsensitized spleen cells could also form protected foci on L-cell monolayers, although the numbers of protected foci formed by these nonsensitized spleen cells were about one-hundredth of those of sensitized spleen cells. We designated these focus-forming cells as natural interferon-producing cells (NIPC). In this communication, we report the nature and the distribution of NIPC in mice.

MATERIALS AND METHODS

Mice. Male C57BL/6, C3H/He, CBA, BALB/c, and BALB/c nude (nu/nu) mice weighing 25 to 30 g were used in the present study. Specific-pathogen-free BALB/c and congenitally athymic nude (nu/nu) mice derived from BALB/c mice were purchased from the Central Laboratory of Experimental Animals (Tokyo) and maintained in the isolators of our laboratory.

Germfree ICR mice and their corresponding conventional mice were originally obtained from the Central Laboratory of Experimental Animals (Tokyo) and had been maintained by successive brother-sister matings in our laboratory.

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. The culture medium for mouse lymphoid cells was MEM supplemented with 5% fetal calf serum.

Interferon assay. Interferon was assayed by the cytopathic effects inhibition microassay method previously described (7) by using L cells and VSV as the challenge virus. One interferon unit in our laboratory was equivalent to 2.7 international reference units of mouse interferon.

Enumeration of NIPC in mice. Whole spleens, mesenteric lymph nodes, and thymuses were removed aseptically from untreated mice and teased on steel mesh immersed in chilled MEM in a plastic dish. The cells which passed through were washed twice with medium, and the cell suspensions were prepared. Resident peritoneal cells were used as unstimulated peritoneal cells. These cells were cocultivated with L-cell monolayers in plastic dishes (catalog no. 3002, Falcon Plastics). At 3 h after cocultivation, the 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 each of these monolayers and allowed to gel in a cold room for 5 min. Then, the L cells were incubated at 35°C for another 12 h, after which the agar was removed; the cells were washed carefully

with MEM to remove all traces of agar and inoculated with about one plaque-forming unit of VSV per cell. After the 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 the control monolayers were completely lysed. After vigorous washing to remove dead cells, the remaining cells were stained with May Gruenwald-Giemsa solution. The focus counting was checked by microscopic observation.

Separation of spleen cells into glass-adherent and nonadherent cells. The method for separating spleen cells into glass-adherent and nonadherent cells was described previously (1). The spleen cell suspension (10^6 cells per ml) was distributed into a number of glass tissue culture dishes (60 by 15 mm), 4 ml per dish, and incubated at 37°C for 2 h. After incubation, the supernatant fluid containing nonadherent cells was aspirated from each dish and transferred to a second set of dishes. The first set of dishes retaining mostly adherent cells were refilled with fresh MEM. Both were again incubated at 37°C for 2 h. After incubation, the supernatant fraction of the second dishes which contained nonadherent cells was gently aspirated. Nonadherent cells in the supernatant portion were collected by centrifugation and washed repeatedly with MEM. Adherent cells remaining in the dishes were repeatedly washed by flushing with MEM, and then the cells were suspended in MEM by scraping them off with a rubber policeman. Before use, cell counts were made on May Gruenwald-Giemsa-stained smears of each cell suspension.

Antisera. Anti-mouse immunoglobulin serum was obtained from the Fujizoki Pharmaceutical Co., Tokyo, Japan. Anti-H-2^b and anti-Ia (I region-associated antigens 8 and 9) antisera were purchased from Searle Diagnostic Co., Bucks, England. Anti-Thy 1.2 antiserum was prepared by the method of Takahashi et al. (10).

RESULTS

Protected focus formation by peritoneal cells or spleen cells derived from untreated C57BL/6 mice. In the first experiment, peritoneal cells derived from untreated C57BL/6 mice were cocultivated with L-cell monolayers in liquid cultures for 1, 2, 3, or 5 h, and then the assay for protected focus-forming cells was performed. The number of protected foci (ca. $26/2 \times 10^5$ cells) was independent of cocultivation time in liquid cultures (data not shown). In the following experiment, untreated mouse cells were cocultivated in liquid culture with L-cell monolayers for 3 h. There was a consistent linear relationship between the numbers of unstimulated peritoneal cells applied on L-cell monolayers and the focus counts in a dilution series (Fig. 1).

Subsequently, we performed experiments to investigate whether or not the protected focus formation was mediated by interferon. Since cycloheximide was known to inhibit both the production and the action of interferon, we examined the effect of the drug on the formation of the protected foci of L cells.

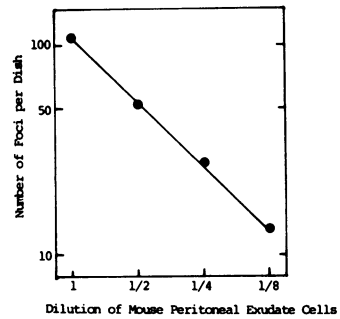

FIG. 1. *Effects of unstimulated peritoneal cell numbers upon focus formation. Serial twofold dilutions of peritoneal cells were cocultured with L-cell monolayers. The starting number of peritoneal cells was 10^6.*

After cocultivation of unstimulated peritoneal cells with L cells 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 the monolayers were washed three times with MEM and inoculated with VSV. The formation of the protected foci was completely blocked by inhibition of protein synthesis (data not shown). No foci were observed when unstimulated peritoneal cells were overlaid on the monolayers of FL cells, whose origin is human amnion (data not shown). To gain more direct evidence that focus formation was mediated by interferon, the effect of anti-interferon (type I) antiserum on focus formation was investigated. When anti-interferon antiserum was added to agar after cultivation in liquid cultures, there was a decrease in focus counts of about 80% (Table 1). These results indicate that the formation of protected foci by unstimulated mouse spleen cells is mediated for the most part by type I interferon that was produced without the addition of a special inducer. In the contrary, the formation of protected foci by IIPC was mediated by type II interferon (2). Thus, the cells which produced interferon in this system were designated as NIPC.

About 20% of the foci of which formation was not inhibited by anti-type I interferon antiserum might be related to the production of type II interferon or some other virus inhibitor(s) by unstimulated mouse spleen cells.

Nature of NIPC. To examine which cell type of the spleen is responsible for the protected focus formation, we divided mouse spleen cells into two fractions according to glass adherence, and each fraction was tested for focus-forming capacity. Unfractionated spleen cells and the glass-adherent fraction could form protected foci on L-cell monolayers (29 or 54 per 10^6 cells,

TABLE 1. *Effect of anti-interferon (type I) antiserum on focus formation*[a]

Source of spleen cells	No. of foci[b]	
	No treatment	Anti-IF antiserum[c] treatment
L-cell-sensitized C57BL/6 mice[d]	283 ± 16	269 ± 2
Untreated C57BL/6 mice	103 ± 14	22 ± 7

[a] Spleen cells from L-cell-sensitized (2.5×10^5) or untreated C57BL/6 mice (10^7) were cocultivated with L-cell monolayers for 3 h. L-cell monolayers were washed, and then soft agar containing 1% antiserum was poured onto them. After 12 h, the agar was removed, and the cells were washed three times with MEM and inoculated with VSV.

[b] Mean ± standard deviation of the mean.

[c] Anti-type I rabbit serum (anti-interferon titer 1: 128×10^2). This antiserum was donated by S. Kohno, National Institute of Health, Tokyo.

[d] C57BL/6 mice were preinjected intraperitoneally with L cells (10^7) 7 days previously. When L-cell-sensitized spleen cells were cocultivated with L-cell monolayers for 24 h, immune interferon could be detected in the culture fluids.

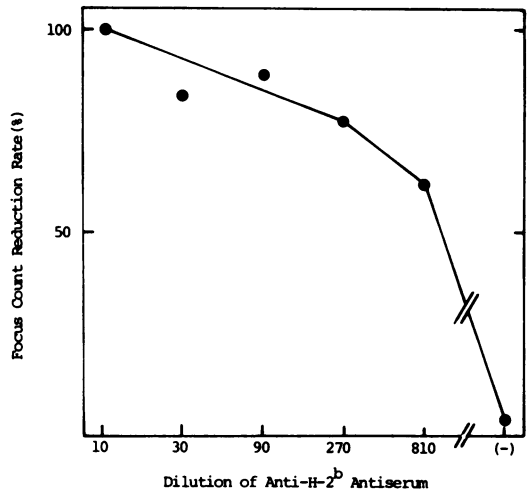

FIG. 2. *Effects of anti-H-2^b antiserum on focus formation. Peritoneal cells derived from untreated C57BL/6 mice were cocultivated with L-cell monolayers for 3 h. L-cell monolayers were washed and then treated with threefold-diluted anti-H-2^b (B10Br anti-C57BL/10ScSn) antiserum for 30 min, and soft agar was poured onto these monolayers. After 12 h, the agar was removed, and the cells were washed three times and inoculated with VSV.*

respectively). On the other hand, the non-glass-adherent fraction could form only a few focus counts ($2/10^6$ cells). These findings suggest that the NIPC may belong to the macrophage population.

In the next experiment, cell surface antigen(s) of NIPC was directly determined by means of treatment of unstimulated peritoneal cells with specific antibody plus complement after cocultivation with L-cell monolayers in liquid culture for 3 h; that is, just before being overlaid with soft agar. Treatment with anti-Thy 1.2 antiserum, anti-immunoglobulin antiserum or anti-Ia antiserum plus complement showed no effect on focus formation (data not shown). H-2 antigen(s) was expressed on the surface of NIPC (Fig. 2).

Organ distribution and mouse strain distribution of NIPC. All of the above studies were performed with spleen cells or peritoneal cells. It was of interest to examine whether this reactivity was limited to these organs or was also present in other ones. Peritoneal cells, spleen cells, and bone marrow cells derived from untreated C57BL/6 mice contained a large number of NIPC (1076, 173, and 306 per 10^7 cells, respectively). On the other hand, no NIPC could be demonstrated in thymocytes or mesenteric lymph node cells. The distribution of NIPC seemed to be similar to that of macrophages (12).

In the next experiment, whether NIPC were distributed in mouse strains other than C57BL/6 mice was examined. Mouse strains examined in this experiment were C3H/He, CBA, BALB/c, and congenitally athymic BALB/c nude mice. NIPC were found in the spleen and peritoneal cells of all mouse strains, suggesting that NIPC were widely and generally distributed in mice (Table 2). To determine the nature of NIPC, we studied cells derived from congenitally athymic nude mice. We found that the mice spleen cells from nude mice were relatively rich in NIPC (Table 2).

NIPC and spontaneous interferon production in germfree mice. Since some types of natural immunity are evoked by infection with bacteria or viruses which can induce interferon in vivo and in vitro, it was important to look for NIPC in mice reared under germfree conditions. Germfree mice (ICR strain) had almost as many NIPC as conventionally reared mice (Table 3).

Several investigators reported that peritoneal macrophages could produce interferon without the addition of special inducer (6, 9). The above results and these reports led us to examine the spontaneous interferon production in the germfree mice maintained in our laboratory. Unstimulated peritoneal cells derived from individual conventional or germfree ICR mice (mean numbers of peritoneal cells were 5×10^5 or 7×10^5, respectively) were suspended in 0.3 ml of MEM and were cultured for 2 days. Individual culture fluid was assayed for interferon activity, and there was no difference between that of the

TABLE 2. *Number of focus-forming cells in spleen and peritoneal cells derived from various mouse strains*[a]

Mouse strain	No. of foci[b] per 10^7 cells	
	Spleen cells	Peritoneal cells
C57BL/6	190 ± 25	1,600 ± 95
CBA	97 ± 2	3,013 ± 784
C3H/He	650 ± 15	3,719 ± 393
BALB/c	120 ± 22	6,014 ± 606
Congenitally athymic nude mice (BALB/c strain)	760 ± 239	1,736 ± 167

[a] Spleen cells or peritoneal cells from various mouse strains were cocultivated with L-cell monolayers for 3 h. L-cell monolayers were washed, and then soft agar was poured onto them. Twelve hours later, the agar was removed, and the cells were washed three times with MEM and inoculated with VSV.

[b] Mean ± standard deviation of the mean.

TABLE 3. *Protected focus formation by various cell fractions derived from conventional or germfree ICR mice*[a]

Cell fraction	No. of foci[b] per 10^7 cells	
	Conventional mice	Germfree mice
Spleen cells	179 ± 42	293 ± 17
Peritoneal cells	2,080 ± 164	3,400 ± 280
Bone marrow cells	710 ± 65	92 ± 9

[a] Various cells derived from conventional or germfree ICR mice were cocultivated with L-cell monolayers for 3 h. L-cell monolayers were washed, and soft agar was poured onto them. Twelve hours later, the agar was removed, and the cells were washed three times with MEM and inoculated with VSV.

[b] Mean ± standard deviation of the mean.

conventional and germfree mice (data not shown). Subsequently, a kinetic study using peritoneal cells pooled from eight conventional or eight germfree ICR mice was carried out. Little difference was seen in the kinetics of interferon production and the maximal titers between the interferon of conventional and germfree mice (Fig. 3). Contamination with bacteria could not be detected in this experiment.

DISCUSSION

In the present study, many untreated mice have been found to have protected focus-forming cells on L-cell monolayers. The formation of the protected foci against virus infection was mediated for the most part by type I interferon which was produced autogenously. In a previous study (2), we succeeded in enumerating IIPC, and the focus formation by IIPC on the L cells proved to be mediated by type II interferon.

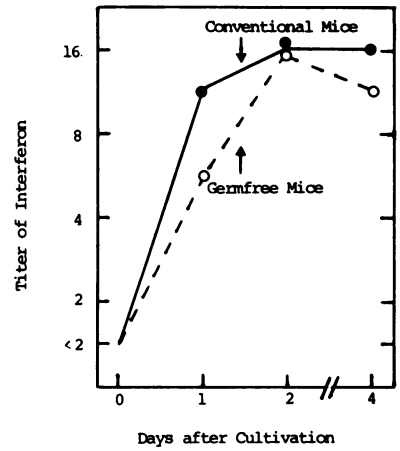

FIG. 3. *Unstimulated peritoneal cells (8 × 10^5) pooled from eight conventional or eight germfree ICR mice were tested for their ability to spontaneously produce interferon. Culture fluid assayed for interferon activity was obtained after 1, 2, and 4 days of incubation in MEM.*

Thus, we have designated these focus-forming cells as NIPC. The glass-adherent fraction could form the protected foci on L-cell monolayers, suggesting that NIPC might belong to the macrophage fraction. Thy 1 antigen, immunoglobulin, and Ia antigen could not be detected, but only H-2 antigen(s) was expressed on the surface of NIPC. This finding was consistent with the hypothesis that NIPC might belong to the Ia negative macrophage fraction. NIPC were found in spleen, bone marrow, and peritoneal cells. The tissue distribution of NIPC agrees with that of macrophages which was reported by Yokochi et al. (12). From the finding that NIPC were detected in congenitally athymic nude mice, it was concluded that NIPC were not mature T cells.

Trinchieri et al. (11) have recently reported that interferon is released in the supernatant of mixed cultures of nonsensitized lymphocytes and certain tumor-derived and virus-transformed cells. In our study, no interferon activity was found in the supernatant of cocultures between nonimmunized spleen cells and L cells, even after a long incubation period (7 to 10 days) (unpublished data). The possibility, however, remains to be excluded that interferon is not really produced spontaneously but is induced by a small portion of lymphoid cells through contact with L cells.

Since bacteria and bacterial products are known to induce in vivo and in vitro formation of interferon, germfree animals can be utilized as more refined test animals for investigations involving the spontaneous interferon production.

Our results indicate that mouse lymphoid cells derived from germfree mice could form the protected foci on L-cell monolayers; that is, they could produce interferon without the addition of a special inducer irrespective of their history of preexposure to bacterial products. The presence of low levels of interferon-like activity in media from unstimulated cell cultures or in extracts of animal tissues has been described in several studies (6, 9). It was reported that rabbit peritoneal macrophages placed in phosphate-buffered saline at 37°C produced interferon without receiving any inducer (5). It was shown that when the peritoneal macrophages of an intact rat were placed in saline solution, interferon was emitted into fluid, but interferon was not released from the peritoneal macrophages of a germfree rat (5). DeMayer et al. also reported the same result in germfree mice (1). Under our experimental conditions, however, peritoneal exudate cells derived from germfree ICR mice spontaneously produced, when cultured in vitro, interferon. This discrepancy is inexplicable at present. Contamination of bacteria could not be detected in the ICR germfree mice used in this study. When sera from 51 germfree ICR mice were examined for reactivity against Sendai, reo 3, Theiler's GD VII, ectromelia, and mouse hepatitis virus antigen, there was no positivie reaction against all of the antigens tested (8). Moreover, the attempt of virus isolation from homogenates of various organs including lungs, livers, kidneys, and intestines of 6 germfree ICR mice was unsuccessful (8). Contamination with a very small amount of endotoxin in our culture system could not be completely ruled out.

NIPC are considered to be a cellular background for spontaneous interferon production. From this study, it could be concluded that only 2 to 8 cells per 10^4 macrophages spontaneously produced interferon within 15 h under our experimental conditions. Whether this small portion of macrophages belongs to a specific subpopulation remains to be determined. Further studies on the specific role of NIPC in vivo are to be undertaken.

Natural killer (NK) activity can be augmented by interferon, and NK cells can produce interferon (4). It is interesting that athymic nude mice were found to have a high level of NIPC while they are also known to have high NK activity. The relationship between NIPC and NK cells is worth investigating.

ACKNOWLEDGMENTS

The technical assistance of E. Iwata and T. Tsuruguchi is greatly appreciated. S. Kohno, National Institute of Health, Tokyo, generously supplied the antiserum against type I interferon (L cell-NDV).

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