

Interferon-Producing Capacity of Germfree Mice

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The general capacity of germfree mouse spleen cells to produce interferon *in vitro* in response to various stimuli was investigated. The interferon response of germfree mouse spleen cells *in vitro*, when compared with that of the conventionals, appears to be lower to some inducers. Interferon production *in vitro* stimulated by hemagglutinating virus of Japan (HVJ) or BHK-HVJ cells (BHK cells persistently infected with HVJ) was apparently suppressed in germfree mouse spleen cells as compared with the corresponding conventionals, whereas no difference of interferon production was observed between germfree and conventional mouse spleen cells in response to Newcastle disease virus, *Escherichia coli* endotoxin, poly(I:C), and phytohemagglutinin. Although monocontamination with HVJ had no enhancing effect on the interferon-producing ability of germfree mouse spleen cells in response to HVJ, conventionalization for 2 weeks greatly enhanced interferon-producing capacity.

Germfree animals play important roles in investigations that would otherwise become complicated by the presence of a microbial flora. Since bacteria and bacterial products are known to induce the *in vivo* formation of interferon, it seems likely that the germfree animal can be utilized as a more refined test animal for investigations involving the *in vivo* induction and production of interferon using bacterial stimuli.

De Somer and Billiau (2) first reported that, when the spleen interferon levels of germfree rats were measured 2 h after inoculation with heat-killed *Escherichia coli* and compared with those of conventional rats, there was no difference at all. Fitzgerald and Pollard (3) also reported that the time pattern of serum interferon response to Newcastle disease virus (NDV) stimulation in germfree CFW mice seemed to be essentially the same as that in conventional animals. However, Considine and Starr (1) showed, that although high interferon yields were obtained in germfree SW mice compared with corresponding conventional mice when stimulated by NDV, the germfree mice, stimulated by mouse hepatitis virus, showed a smaller interferon response. As noted above, experimental results obtained from works dealing with the interferon-producing capacity of germfree animals have been inconsistent.

In this study, the general capacity of germfree mouse spleen cells to produce interferon

in vitro in response to various stimuli was investigated.

MATERIALS AND METHODS

Animals. The mice used in this study were germfree ICR and C3H mice and their corresponding conventional mice, which were originally obtained from the Japanese Central Institute of Experimental Animal Research and had been maintained by successive brother-sister matings in our laboratory. They were used at about 6 weeks of age and each experimental group was made to consist of an approximately equal number of males and females.

Spleen cell suspension. Germfree or conventional mouse spleens were teased on steel-mesh immersed in chilled TC-199 medium contained in a plastic dish, and cells that passed through were washed twice in the medium by repeated centrifugation and suspension. Before use, cell counts were performed and splenograms were made on May-Grünwald-Giemsa-stained smears.

Interferon production. Interferon inducers used in this study were NDV (Miyadera strain), hemagglutinating virus of Japan (HVJ; Nagoya strain), BHK-HVJ cells (BHK cells persistently infected with HVJ) (5, 6), poly(I:C)(Maryland), phytohemagglutinin-*p* (PHA-*p*)(Difco), and *E. coli* endotoxin (Difco). NDV and HVJ were used at multiplicities of infections of 10, poly(I:C) at 25 $\mu\text{g/ml}$, PHA-*p* at 0.01 ml/ml, and endotoxin at 100 $\mu\text{g/ml}$. As described in the previous paper (6), more than 80% of the BHK-HVJ cells exhibited a hemadsorbing capacity in most passages, and the viral products released from the cells showed 4 to 8 hemagglutinating units/0.25 ml, but the greatest part was non-infectious component.

Spleen cell suspension prepared from one mouse was divided into halves and incubated separately with a different interferon inducer in TC-199 medium.

After an appropriate period of incubation, the culture fluid was clarified by centrifugation at 2,000 rpm, and then the supernatant fluid was stored at -20 C until assayed for interferon. To eliminate infectious virus, interferon samples induced by NDV or HVJ were centrifuged at 20,000 rpm for 60 min, dialyzed overnight at pH 2.0, and redialyzed to neutrality against Hanks solution.

Interferon titration. Interferon was assayed by the plaque reduction method with mouse L cells and vesicular stomatitis virus as challenge virus (5). Individual fluid were then diluted serially in twofold steps in maintenance medium; 3.0-ml aliquots of each dilution were placed on triplicate cultures of L cells in petri dishes and incubated at 35 C for 24 h. The cultures were drained and challenged with about 100 plaque-forming units of vesicular stomatitis virus. After 1 h of incubation at 35 C, the culture was overlaid with 1% agar in YLE. On day 2 of virus challenge, the L-cell monolayer was stained with neutral red and plaques were counted. Titers of interferon were expressed as the reciprocals of dilutions causing a 50% plaque count reduction.

RESULTS

Splenogram. Cell counts were performed on each spleen cell suspension prepared from a whole spleen, and numbers of free cells in the spleen were estimated. Differential counts (splenogram) were made on a May-Grünwald-Giemsa-stained smear of each suspension from germfree, conventionalized (2 weeks after release from germfree isolation), and conventional ICR mouse.

Table 1 shows the total numbers of free spleen cells and splenograms. There was little difference among germfree, conventionalized, and conventional ICR mice in the total numbers of spleen cells. However, percentage of pyroniophilic cells was low in the germfree mouse spleen and macrophage frequency was highest in the conventional mouse spleen.

Interferon production by various inducers. Spleen cells obtained from germfree and conventional ICR mice were tested in vitro for their ability to produce interferon in response to various inducers, such as NDV, HVJ, BHK-HVJ cells, *E. coli* endotoxin, poly(I:C), and PHA. When stimulated by NDV, endotoxin, poly(I:C), or PHA, there was no significant difference between the germfree and conventional (Table 2). When stimulated by HVJ or BHK-HVJ cells, however, the germfree did not produce as high titers of interferon as did the conventional (Table 2). To rule out the possibility that the lower interferon titers seen with germfree mouse spleen cells were simply due to different kinetics of interferon production, kinetics studies of interferon production by germfree and conventional mouse spleen cells stimulated with BHK-HVJ cells were carried out. The results are illustrated in Fig. 1. Little difference was seen in the time pattern of interferon response between the germfree and conventional, although the conventional responded with much higher titers of interferon than did the germfree.

To see whether the lower capacity of germfree mouse spleen cells to produced interferon in vitro in response to HVJ or BHK-HVJ cells was restricted to the ICR strain, similar experiments were repeated using C3H mice. The results obtained in C3H mice were essentially the same as those in ICR mice (Table 3). Thus, the observed lower responsiveness of germfree mouse spleen cells to HVJ or BHK-HVJ cells appears to be a common phenomenon among germfree mice.

Effect of HVJ-monocontamination on interferon-producing capacity of germfree mouse spleen cells. Since it is well known that mice are widely contaminated with HVJ, a relatively increased interferon-producing capacity of conventional mouse spleen cells, as compared with the germfree, may be simply due to HVJ contamination and, consequently, to "immuno-

TABLE 1. *Splenogram*" of ICR mice

Spleen type	Cell counts ($\times 10^7$)	Differential (%)			
		Lymphoid cells	Pyroniophilic cell ^b	Macrophages	Others
Germfree	20.5 \pm 2.5	96.9 \pm 2.5	1.5 \pm 0.5	2.7 \pm 2.5	0.5 \pm 0.5
Conventionalized ^c	23.2 \pm 2.5	97.3 \pm 1.0	8.0 \pm 4.0	1.8 \pm 1.0	0.5 \pm 0.5
Conventional	18.2 \pm 3.8	92.8 \pm 3.5	7.2 \pm 3.2	7.7 \pm 5.0	1.2 \pm 1.0

^a A splenogram was made on a May-Grünwald-Giemsa-stained smear of each suspension from germfree, conventionalized, and conventional ICR mice (mean of five mice).

^b The lymphoid cell with a deeply basophilic cytoplasm.

^c Conventionalized, Released from germfree isolator. Splenograms were performed 2 weeks after release from the isolator.

TABLE 2. *In vitro* interferon production by ICR mouse spleen cells^a

Inducer	Hours after induction	Individual titer ^b	
		Germfree	Conventional
NDV (10 MOI)	24	3.7, 10, 3, 7.5, 7.5 (6.3)	5, 6, 6, 10 (6.8)
Endotoxin (100 μ g/ml)	12	5, <2, 2.9, <2, <2 (1.6)	4.2, <2, 5, 3.2, <2 (2.5)
Poly(I:C) (25 μ g/ml)	23	3.8, 3.3, <3, <3, 3, <3 (1.7)	19, 4.3, 7.5, <3, <3 (6.1)
PHA (0.01 ml/ml)	24	3.2, 3.9, 3.7, 3.9, 5.2, 4.9, 4.2 (4.1)	4.3, 5, 4, 6.8, 3.7, 5, 15.9 (6.4)
HVJ (10 MOI)	24	7,600, 8,000, 2,100, 1,500, 4,500 (4,740) ^c	16,000, 17,000, 16,000, 19,000 (17,000) ^c
BHK-HVJ cells ^d (5×10^6 cells)	24	97.4, 278, 194, 115, 254, 245 (197.2) ^c	841, 1,026, 1,013, 964, 788 (966.4) ^c

^a The culture fluid was clarified by centrifugation, and the supernatant fluid was stored at -20 C until assayed for interferon. MOI, Multiplicity of infection.

^b Interferon titers were assayed by the plaque inhibition method using L cells and vesicular stomatitis virus. Titters of interferon are expressed as the reciprocals of dilutions causing 50% plaque count reduction.

^c This difference is statistically significant ($P < 0.001$, t test).

^d Spleen cell suspension was co-cultivated with BHK-HVJ cells. Details of the method have been published previously (5).

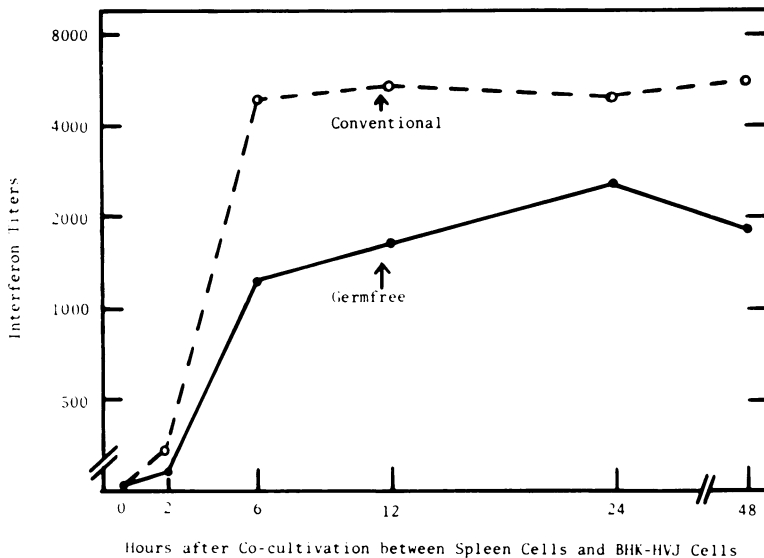


FIG. 1. Spleen cells obtained from conventional or germfree mice were tested *in vitro* for their ability to produce interferon in response to BHK-HVJ cells. Culture fluid was obtained after 2, 6, 12, 24, and 48 h of co-cultivation with BHK-HVJ cells and assayed for interferon. Interferon titers are expressed as the reciprocals of dilutions causing 50% plaque count reduction.

induction." To test this possibility, germfree ICR mice were intranasally monocontaminated with HVJ, and 2 weeks later their spleen cells were tested for the ability to produce interferon *in vitro* in response to HVJ stimulus. Local pulmonary consolidation was invariably observed in monocontaminated ICR mice. The results shown in Table 4 indicate that monocontamination of germfree ICR mice with HVJ has no enhancing effect on interferon-producing capacity of their spleen cells in response to

HVJ. On the contrary, it appears to suppress the capacity, even though the difference between the mean titers is not very significant.

Enhancements of *in vitro* interferon-producing capacity of germfree mouse spleen cells by conventionalization. Two weeks after release from germfree isolation (conventionalization), *in vitro* interferon-producing capacity of spleen cells from these conventionalized ICR mice was tested, using HVJ as inducer, and compared with that of the germfree. The

TABLE 3. *In vitro* interferon production by C3H mouse spleen cells^a

Inducer	Hours after induction	Individual titer ^b (Mean titer)	
		Germfree	Conventional
HVJ (10 MOI)	24	360, 399, 390, 381, 438 (393) ^c	565, 700, 539, 700, 613 (623) ^c
BHK-HVJ cells (5 × 10 ⁶ cells)	24	299, 188, 364, 226, 627 (340) ^c	376, 868, 1,253, 940, 2,820 (1,251) ^c

^a The culture fluid was clarified by centrifugation, and the supernatant fluid was stored at -20 C until assayed for interferon. MOI, Multiplicity of infection.

^b Interferon titers were assayed by the plaque inhibition method using L cells and vesicular stomatitis virus. Titers of interferon are expressed as the reciprocals of dilutions causing 50% plaque reduction.

^c This difference is statistically significant (*P* < 0.001, *t* test).

TABLE 4. *Effects of monocontamination with HVJ on in vitro* interferon production by germfree ICR mouse spleen cells (HVJ, 24 h)

Spleen cells	Individual titers ^a
Germfree	960
	940
	720
	1,830
	1,810
Germfree (HVJ) ^b	580
	710
	570
	580
	770

^a Interferon titers were assayed by the plaque reduction method using L cells and vesicular stomatitis virus. Titers of interferon are expressed as the reciprocals of dilutions causing 50% plaque count reduction. Mean titers: germfree, 1,252; germfree (HVJ), 642.

^b Germfree ICR mice were intranasally monocontaminated with HVJ in a germfree isolation, and 2 weeks later their spleen cells were tested for ability to produce interferon *in vitro* in response to HVJ stimulus.

interferon-producing capacity of spleen cells was apparently enhanced by conventionalization (Table 5). However, HVJ infection superimposed on conventionalization enhanced no more than conventionalization alone.

DISCUSSION

The present study shows that interferon response of germfree mouse spleen cells *in vitro*, when compared with that of the conventionals, appears to be lower in some particular inducers. When stimulated by NDV, *E. coli* endotoxin, PHA, or poly(I:C), there was no difference between the germfree and conventionals. However, interferon production stimulated by HVJ or BHK-HVJ cells was apparently lowered in germfree mouse spleen cells

TABLE 5. *Effects of conventionalization^a and HVJ superinfection^b on in vitro* interferon production by ICR mouse spleen cells (HVJ, 24 hr)

Spleen cells	Individual titer ^c
Germfree	7,100
	7,100
	2,000
	4,200
	4,650
Conventionalized ^a	14,400
	2,400
	58,000
	88,000
	22,500
Conventionalized (HVJ)	28,000
	18,000
	36,000
	54,000
	48,000
	14,200

^a Two weeks after release from germfree isolation (conventionalization), *in vitro* interferon-producing capacity of spleen cells was tested using HVJ as inducer.

^b Conventionalized ICR mice were intranasally infected with HVJ, and 2 weeks after infection *in vitro* interferon-producing capacity of spleen cells was tested.

^c Interferon titers were assayed by the plaque inhibition method using L cells and vesicular stomatitis virus. Titers of interferon are expressed as the reciprocals of dilutions causing 50% plaque count reduction. Mean titers: germfree, 5,010; conventionalized, 31,060; conventionalized (HVJ), 36,040.

as compared with that in the corresponding conventionals.

Glasgow (4) reported that cultures of leukocytes obtained from mice immunized with Chikungunya virus produced 2- to 10-fold greater amounts of interferon when exposed to

an inoculum of Chikungunya virus than similar cell preparations from nonimmune, control animals. Therefore, the observed higher interferon yield from conventional mouse spleen cells, relative to that from the germfree, might be thought to have resulted from a similar immune mechanism. However, this assumption is least likely, since monocontamination with HVJ had no enhancing effect on the interferon-producing ability of germfree mouse spleen cells in response to HVJ. Conversely, the mechanism involved in reduced response of germfree mouse spleen cells to HVJ or BHK-HVJ cells is also unclear. However, considering that conventionalization for 2 weeks greatly enhanced interferon-producing capacity and concurrently increased lymphoid cells with basophilic cytoplasm of the spleen, it is quite likely that these activated cells (immunoblast) may be primary participants in interferon response to a particular stimulus such as HVJ or BHK-HVJ cells.

Endotoxin-induced or endotoxin-type interferon has been considered as "preformed" interferon. In a previous study (unpublished data), we found that peritoneal macrophages of conventional rats, when cultured in vitro, spontaneously released 8 to 64 units of interferon, whereas those of germfree rats failed to release measurable amounts of interferon. Hence, it was inferred that the presence of the normal microbial flora might be a prerequisite for the spontaneous release of (preformed) interferon by peritoneal macrophages. How-

ever, the present result that the spleen cells of germfree mice, in response to endotoxin, appeared to have produced interferon at almost full capacity compared to the conventionals, may indicate that endotoxin could stimulate de novo synthesis of interferon in spleen cells irrespective of their history of pre-exposure to bacterial products.

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