ing chiefly the core polysaccharide, was retained by the liver while ^{125}I, labeling chiefly lipid A, was rapidly excreted by the kidney, stomach, and salivary glands probably as free iodide. Stomach slices, but not liver or spleen slices, split ^{125}I from labeled endotoxin *in vitro* suggesting that the stomach may play a role in endotoxin degradation. The radioactivity of ^{125}I labeled endotoxin was excreted more rapidly by immunized than nonimmunized animals.

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Studies on Early-Appearing Interferon *in Vitro*
I. Production of Endotoxin-Induced Interferon by Mouse Spleen Cells Cultured *in Vitro* (33908)

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It has been demonstrated that at least two kinds of interferon (IF) appear in plasma of intact animals by injections of endotoxin (ET) or virus (1). The ET produces peak levels of plasma IF about 2 hr after injection. In contrast, virus-induced IF reaches its maximum titers at 6–12 hr after injection of the inducer. The former and the latter are called the early-appearing IF (early-IF) (2, 3) and the late-appearing IF (late-IF) or IF (4, 5), respectively.

It is well known that the late-IF is synthesized within the cells in response to the inducer. By using metabolic inhibitors, the above fact was confirmed both *in vivo* and *in vitro* (6–9). On the other hand, in the case of early-IF, it has been observed that its production in rabbit plasma was not affected by the treatments of actinomycin D (10) or puromycin (11). These results have been confirmed in mice (12).

Recently, Smith and Wagner (13) reported that rabbit macrophages cultures *in vitro* could produce ET-induced IF and spontaneous IF without inducer. Nagano *et al.* (14) also demonstrated rapid "sponatneous" release of IF by similar cells *in vitro*.

However, the production of macrophage-IF was inhibited by actinomycin D or puromycin. More recently, Finkelstein *et al.* (15) reported that the production of ET-induced IF by mouse macrophages cultures *in vitro* was relatively resistant to inhibition by actinomycin D. This result was of much interest. However, the molecular basis for its production mechanism is still unclear.

The present paper describes the kinetics and cellular conditions for producing the ET-

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induced early-IF and late-IF by mouse spleen cells cultured *in vitro*. The influences of metabolic inhibitors, actinomycin D and puromycin, on the IF-production are also described.

Materials and Methods. Animals and cells. Spleen cells for IF production were prepared from DDY strain mice, weighing about 20 g. An IF-sensitive line of L cells was used for IF-assay.

Media. Growth media for cultivation of L cells and mouse spleen cells consisted of Eagle's minimum essential medium (EM) [supplemented 10 and 5% bovine serum (BS), respectively] and antibiotics. The EM + 5% BS was used for cultivation of primary chick embryo cells.

Viruses. Newcastle disease virus (NDV) (Miyadera strain) was grown in the allantoic cavity of 10-day-old embryonated eggs. The NDV sotck had 1×10^9 plaque-forming units (PFU)/ml as measured in chick embryo fibroblast monolayer. The stock of vesicular stomatitis virus (VSV) was prepared in chick embryo cell culture, and the infective titer of 6×10^6 PFU/ml as assayed on monolayer of L cells.

Reagents. Actinomycin D was a gift of Dr. Tawara, Institute of Shionogi Co., Ltd. *Escherichia coli.* (055:B5), ET, and other reagents were purchased commercially.

Preparation of mouse spleen cells. All procedures were carried out at 4°. Mouse spleens were gently minced in EM + 5% BS and released cells were washed twice with the same medium. After filtration through 150-mesh net-filter, the suspension was adjusted to the desired cell concentration. Approximately 70, 15, and 15% of the cells in the suspension were small lymphocytes, mononuclear cells, and polymorphonuclear leukocytes, respectively.

IF production in vivo. A 200-μg dose of endotoxin (0.1 ml of the stock solution) was injected intravenously into each mouse. After injection of the inducer into the tail vein, plasma and spleen samples from groups of 10 mice were obtained at different times. Spleen suspended in cold EM were homogenized in a Teflon glass homogenizer. The homogenate was centrifuged at 100,000*g* for 1 hr to remove cell debris. The supernatant was used for spleen-IF *in vivo*.

IF production in vitro. Each of the spleen cell suspensions was transferred into glass petri dishes and incubated at 37° for different periods in an atmosphere of 5% CO_2. In most experiments IF production was induced in cultures that contained 1×10^8 spleen cells/ml, and the dose of added ET was 100 μg/ml. The NDV-induced IF sample induced by about 2 MOI of the virus were centrifuged at 100,000*g* for 1 hr and dialyzed overnight against citrate–HCl buffer, at pH 2, to remove the virus. They were then redialyzed to neutrality against PBS.

IF assay. For the assay of IF titer in L cell or chick embryo fibroblast cultures, the small modified plaque reduction method (16) with VSV was used. The titers of IF samples were expressed as the reciprocal of the dilution that reduced the plaque count to 50% of that of the controls (PRU_{50}/3 ml).

Assay of lysosomal enzymes. The activity of acid phosphatase was determined by a small modification of the method of Bessey *et al.* (17). β-Glucuronidase activity was measured by a modified method of Fishman (18).

Results. Assay system. The effect of residual ET on the IF assay system was tested. Preliminary experiments show that ET even at a concentration of 200 μg/ml failed to show any inhibitory effect on the PFU. It was suggested that the L cells–VSV system could be used for assay of IF activity, if the concentration of ET in the IF preparation was less than 200 μg/ml. The effect of length of treatment with IF on the assay system was investigated, using ET-induced IF from plasma and spleen *in vivo*. The inhibition of pfu reached almost maximum rate at 10 hr after the addition of plasma-IF. A little decrease of inhibition rate was observed 27 hr later. The spleen-IF showed very similar action to that of plasma-IF. On the bases of these findings, it was determined that 20 hr after addition of IF was a suitable time for assay of IF titer by using L cells–VSV system.

Production of ET-induced IF in vivo. Figure 1 shows the production processes of plas-

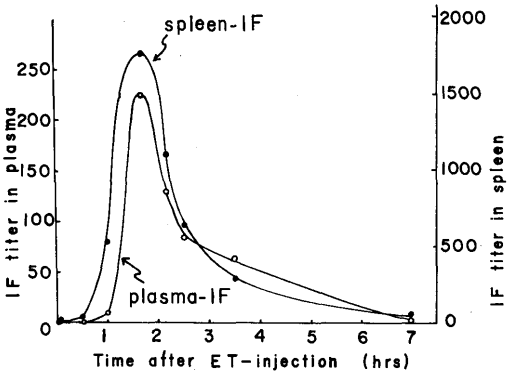

FIG. 1. Appearance of early-IF in mouse plasma and spleen after intravenous injection of ET: the IF titers are the amounts of IF/3 ml of plasma and those /3 g of wet weight of spleen.

ma- and spleen-IF in mice after intravenous injection of ET (200 μg/mouse). In the case of plasma-IF, its titer 1 hr after injection of the inducer was very low. As many others have reported (2, 3), the titer reached its maximum 2 hr later. The peak titer of spleen-IF was also obtained 2 hr after injection of the inducer. However, the production of spleen-IF appeared to occur somewhat more rapidly than plasma-IF; the titer at 1 hr was approximately ⅓ of that at 2 hr. In each case, the titer decreased rapidly after 2 hr and it was scarcely detected at 7 hr.

Appearance of lysosomal enzymes in vivo. It was reported that β-glucronidase, a lysosomal enzyme, appeared in mouse plasma in parallel with the appearance of early-IF after intravenous injection of ET (50 μg/mouse) (19). It appeared that the release of lysosomal enzyme might be related to the production mechanism of early-IF. As shown in Fig. 2, appearance of two kinds of lysosomal enzymes, β-glucronidase and acid phosphatase, was investigated in mouse plasma and spleen after intravenous injection of to that of early-IF appearance in plasma. both enzyme activities showed a peak titer 2 hr after ET injection, and then decreased rapidly. Such phenomena were very similar to that of early-IF appearance in plasma. While considerably higher activities of enzymes in spleen appeared even at 1 hr, and the activities at 2 hr reached the maximum. These appearance of enzyme activities were also very similar to that of early-IF in spleen. Thus, the appearance of early-IF and enzymes in spleen appeared to precede those in plasma. It was suggested lysosome might play any role in the production of early-IF.

Production of ET- or NDV-induced IF in vitro. It was investigated whether spleen cells even *in vitro* can produce early-IF as appeared *in vivo* experiments with ET. Figure 3 shows the production porcesses of IF from spleen cells cultured in EM (+5% BS) or PBS after inoculation of ET (100 μg/ml). The cultured cells in either EM(+5% BS) or PBS did not cause any production of IF until 22 hr. However, the addition of ET caused the production of IF in each cell cultures, although the level of IF titer was very low in comparison to that *in vivo* (see Fig. 1). In both cases, no IF could be detected at 1 hour after ET addition. A very low titer of IF (3 units) appeared in the PBS cultures at 1.5 hr, and the titer was constant between 1.5 and 8.5 hr, whereas in the case of EM cultures, IF activity could be readily detected at 1.5 hr and reached the first peak at 2 hr. The activity decreased slightly until 2.5–3.5 hr, but then it increased rapidly and reached the second peak at 6–8.5 hr. It was

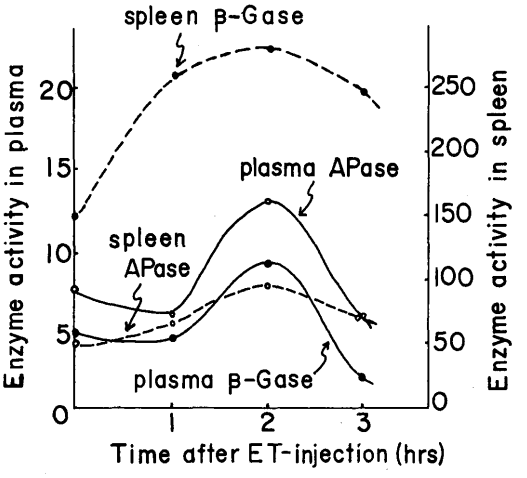

FIG. 2. Appearance of lysosomal enzymes in mouse plasma and spleen after intravenous injection of ET: in the case of acid phosphatase (APase), its activity was expressed as units/0.1 ml (plasma) or 0.1 g(wet wt of spleen)/30 min at 37°. β-Glucuronidase (β-Gase) activity was the same as that of APase except that the reaction time was 17 hr.

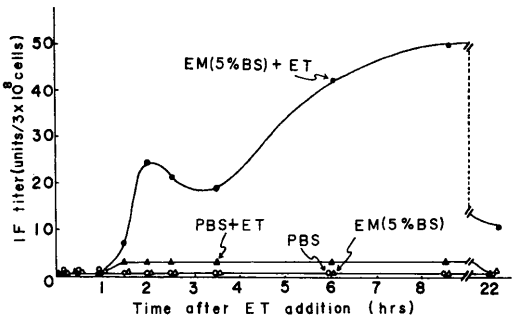

FIG. 3. Appearance of IF in mouse spleen cells cultured *in vitro* after ET addition: after preincubation for 1 hr at 37°, the cultures (3 ml) were exposed to ET (100 μg/ml). The cultures were harvested at each time and centrifuged at 10,000 *g* for 20 min to remove the cells; the supernatant was used as the IF sample.

suggested that two types of IF might be produced by the inducer in the cultured spleen cells *in vitro*. Therefore, the first and second peak's of IF were temporarily referred to the early-appearing interferon (early-IF *in vitro*) and the late-one (late-IF *in vitro*), respectively; 22 hr later, the IF titer decreased remarkably.

From the results shown in Fig. 3, it was inferred that spleen cells cultured in nutrient-rich medium (EM) were able to produce more IF than those in the nutrient-poor solution (PBS). Therefore, healthy cells may be required to produce the IF rather than less-healthy cells. Figure 4 shows the production process of NDV-induced IF in the spleen cells cultured in EM + 5% BS. It reached its peak titer (3000 units) 6 hr after inoculation of NDV. Its peak level continued until 22 hr later. Since the NDV-induced IF was synthesized in the living cells, as described above, these cultured spleen cells seemed to be the living ones.

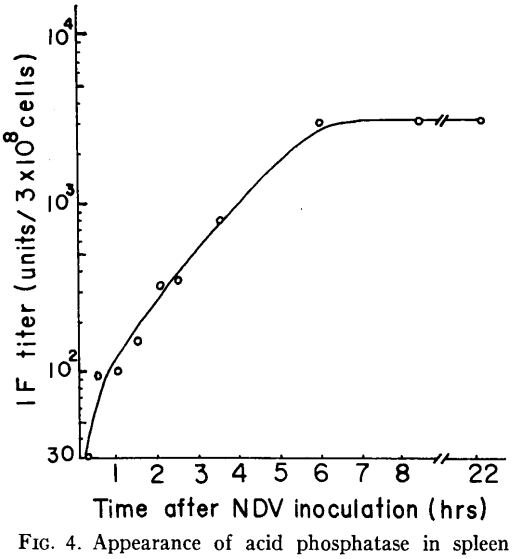

FIG. 4. Appearance of acid phosphatase in spleen cells cultured *in vitro* after addition of ET: the same samples as in Fig. 3 were used to assay the enzyme activity (units/0.1 ml of sample/30 min at 37°).

Appearance of lysosomal enzyme in vitro. The results *in vivo* (Fig. 2) suggested that lysosome might play any role in the production of early-IF. It was of interest to elucidate whether the organella were related to the production of early-IF *in vitro*. Figure 5

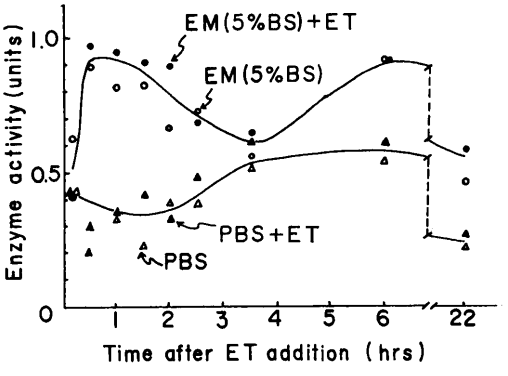

FIG. 5. Appearance of IF in mouse spleen cells cultured in EM (+5% BS) after inoculation of NDV:NDV(2 pfu/cell) were inoculated into the cultures(3 ml) preincubated for 1 hr at 37°. The preparation method of IF is described in "Materials and Methods."

shows the appearance of acid phosphatase in cultured spleen cells after inoculation of ET. The appearance of enzyme activity was observed in EM cultures regardless of the presence or absence of ET. The PBS cultures showed rather low activity and no effect of ET on the release of the enzyme was observed. These results differed remarkably from those *in vivo*.

Properties of mouse spleen IF in vitro. Table I summarized "species-specificities" of mouse IF *in vitro* or *in vivo*. Both the early- and late-IF *in vitro* by ET had small effect

TABLE I. Species-Specificities of Mouse IF *in Vivo* or *in Vitro*.

IF	IF titer (PRU_{50}) L cells	CE cells
ET-plasma early-IF *in vivo*	225	<10
ET-spleen early-IF *in vivo*	1760	<100
NDV-L cell IF[a]	3000	<100
NDV-spleen early-IF *in vitro*	330	<4
NDV-spleen late-IF *in vitro*	3000	<100
ET-spleen early-IF *in vitro*	35	<3
ET-spleen late-IF *in vitro*	45	<7
Chick-IF[b]	<10	190

[a] L cells were incubated with NDV (MOI = 2) and harvested after 20 hr. The harvested culture medium was dialyzed against pH 2, and then redialyzed at pH 7. The dialyzate was centrifuged at 100,000*g* for 1 hr. The supernatant was used as IF sample.

[b] Chick-IF was prepared from the chorioallantoic fluid infected with influenza virus (Type A). The sample was dialyzed at pH 2 and 7, and centrifuged at 100,000*g* for 1 hr to remove the active virus.

on any of the chick embryo cultures; these titers were less than 1/6–1/12 of those in L cells. The ET-induced IF *in vivo* and NDV-induced IF *in vitro* showed the same phenomenon, as is well known. Of course, these chick cells were sensitive to a chick-IF induced by influenza virus, which was not effective in L cells.

It was examined whether early-IF *in vitro* has direct antiviral action. The VSV as mixed with the respective IF and incubated for 70 min at 37°. The early-IF *in vitro* by ET showed no loss in pfu of VSV as was done by NDV-induced IF obtained from L cells or spleen cells.

Table II shows the heat-stabilities of mouse IF. The NDV-induced IF from L cells was not inactivated by heating at 56° for 1 hr, whereas the titer of NDV-induced spleen-IF *in vitro*, either after 2 or 6 hr, was decreased to about 1/6 of the initial titer by the heating. Both the early- and late-IF *in vitro* by ET showed about the same phenomenon.

TABLE II. Heat-Stabilities of Mouse IF *in Vitro*.

IF	IF titer (PRU_{50}) Untreated	(56°, 1 hr)
NDV-L cell IF	3000	3000
NDV-early IF	330	60
NDV-late IF	3000	500
ET-early IF	35	3
ET-late IF	45	10

Table III shows the effects of pH 2.5-treatment and dialysis at pH 7.2 on the spleen-IF *in vitro*. In comparison with NDV-induced IF in L cells, both early- and late-spleen-IF *in vitro* were a little affected by dialysis at pH 7.2 for 16 hr; both titers decreased to about 2/3 of the controls. Although these reductions were observed, they seemed to be generally nondialyzable. The treatment at pH 2.5 for 16 hr had no effect on both spleen-IF *in vitro*, the titers were almost the same as those by dialysis at pH 7.2.

TABLE III. Effects of pH 2.5-Treatment and Dialysis on Mouse Spleen-IF *in Vitro*.

IF	IF titer (PRU_{50}) Untreated	(pH + dial)[a]	Dialysis[b]
NDV-L cell IF	3000	3200	3000
ET-early-IF	35	25	20
ET-late-IF	60	35	35

[a] Treated with pH 2.5 at 4° for 16 hr and dialyzed in PBS (pH 7.2) at 4° for 16 hr.

[b] In PBS (pH 7.2) at 4° for 16 hr.

Effect of actinomycin D on the production of mouse spleen-IF in vitro. Treatment with actinomycin D at a concentration of 10 μg/ml for 1 hr prior to viral infection is known to be quite sufficient to cause complete inhibition of NDV-induced IF in cell cultures (13). Table IV shows the effects of actinomycin D on the production of mouse spleen IF *in vitro*. No IF was produced by spleen cells cultured *in vitro*, without added inducers, at 37° for 2 or 6 hr. However, with the actinomycin treatment, IF-like titer appeared in the medium of cultures without inducers after incubation for 6 hr, although it did not appear at 2 hr. In the case of ET-induced IF, actinomycin D had little or no effects on the production of early-IF at 2 hr, but that of

TABLE IV. Effect of Actinomycin D on the Production of Mouse Spleen-IF *in Vitro*.

Inducer	After induction (hr)	Actinomycin D[a] (μg/ml)	IF titer (PRU_{50}) Exp I	Exp II
None[b]	2	—	<2	<4
		1	—	<4
		10	2	<4
	6	—	<2	<4
		1	—	<4
		10	15	24
ET (100 μg/ml)	2	—	15	11
		1	—	22
		10	14	9
	6	—	47	80
		1	—	26
		10	20	16
NDV (2 MOI)	2	—	420	130
		1	—	120
		10	10	30
	6	—	2800	5600
		1	—	4400
		10	50	55

[a] The spleen cultures were exposed to actinomycin D for 1 hr. After washing the cells, inducers were inoculated into the cultures.

[b] The dialyzates of spleen culture medium against pH 7; the preparation and assay of IF were the same as described in "Materials and Methods."

late-IF at 6 hr was fairly inhibited by the drug treatment (60–80% inhibition). As shown in Table IV, since IF-like activity appeared in the culture medium, without inducer, by only actinomycin D-treatment, the most residual titer of late-IF was supposed to be caused by the actinomycin D-induced IF-like viral inhibitor. Therefore, the production of real IF appeared to be almost completely inhibited by actinomycin D. In the case of NDV-induced IF, actinomycin D inhibited remarkably its production at 2 or 6 hr after the addition of inducer.

Effect of puromycin on the production of mouse spleen IF in vitro. It has been reported that puromycin at 10–50 μg/ml efficiently suppresses the production of rabbit macrophage interferon *in vitro* (13). Table V shows the effects of puromycin on the production of mouse spleen IFs *in vitro*. Puromycin at concentration less than 10 μg/ml appeared to cause incomplete inhibition of the production of both early-and late-IFs *in vitro* by ET. However, 50 μg/ml of puromycin inhibited markedly the both productions. NDV induced IFs at 2 or 6 hours were also considerably inhibited.

Discussion. Data from experiments using actinomycin D or puromycin, suggest that preformed IF is present in significant concentrations in intact animals (10–12). It appeared that ET caused the release of IF from the cells. However, its induction mechanism is still unknown. Sauter and Gifford (19) reported ET-induced circulating IF, early-IF, appeared in parallel to appearance of a lysosomal enzyme, β-glucuronidase, in mouse plasma after injection of ET. This result suggested lysosome alteration associated with ET may play a role in producing the early-IF. However, such phenomena were observed

TABLE V. Effect of Puromycin on the Production of Mouse Spleen-IF *in Vitro*.

Inducer	After induction (hr)	Puromycin[a] (μg/ml)	IF titer (PRU_{50}) Exp I	Exp II
None[b]	2	—	<3	<4
		10	—	<4
		50	<3	<4
	6	—	—	<4
		10	—	<4
		50	—	<4
ET (100 μg/ml)	2	—	18	11
		10	—	5
		50	<3	<4
	6	—	—	80
		10	—	3
		50	—	<4
NDV (2 MOI)	2	—	700	130
		10	—	36
		50	9	24
	6	—	—	5600
		10	—	130
		50	—	4

[a] The spleen cultures were exposed to puromycin for 1 hr, and were incubated with inducers.

[b] The same sample as in Table IV. The preparation and assay of IF were the same as described in "Materials and Methods."

only in intact animals, no evidence at the cell-level *in vitro* has been obtained.

The present results provide some detailed information about the production of early-IF by using intact mouse or its spleen cells cultured *in vitro*. From the kinetic data of IF-production *in vivo* (Fig. 1), it was demonstrated that the ET-induced early-IF in plasma appeared with a small delay compared to that in spleen, although those peak titers were observed at 2 hr after inducer injection. This may suggest the spleen cells are one of the major source of early-IF in plasma, as reported by the other workers (20, 21). Moreover, lysosomal enzymes, β-glucuronidase and acid phosphatase, appeared in parallel to the production of early-IF in plasma or spleen (Fig. 2). These *in vivo* results suggest that lysosomes may play a significant role in the production of early-IF. However, such phenomenon was not observed at the cultured spleen cells *in vitro* (Fig. 5). The release of lysosomal enzyme seems to be unrelated to the IF production.

From the kinetic results *in vitro*, it was suggested that at least two types of IF might be produced in the cultured mouse spleen cells by ET. The spleen cells in the nutrient-rich medium (EM + 5% BS) produced the early-IF at 2 hr and late-IF at 6–8 hr after inoculation of ET (100 μg/ml), although these titers were much lower than those *in vivo* (Fig. 3). However, the spontaneous IF, such as macrophage-IF *in vitro* reported by Smith and Wagner (13) or Nagano *et al.* (14), was not observed at all in the cultured spleen cells without inducer. The cells in nutrient-poor solution (PBS) produced little IF even in the presence of ET. In the present experiments, an IF-producing cell other than macrophages may exist in the cultured spleen cell preparation. In fact, about 70% of the cells in the present preparation were small lymphocytes and only about 15% were macrophages.

Both the early- and late-IF induced by ET *in vitro* possessed "species specificities" and showed no extracellular neutralization of VSV. They were largely inactivated by heating at 56° for 1 hr, stable at pH 2.5 for 16 hr, and generally nondialyzable. These properties were very similar to that of early-IF *in vivo* by ET.

It is of much interest that actinomycin D has little or no effect on early-IF *in vitro* by ET, but puromycin has much effect on it. Smith and Wagner (13) have reported that the production of rabbit macrophage-IF *in vitro* is almost completely inhibited by the treatment with each of the two drugs. This data was not consistent with the idea that the "preformed-IF" required no synthesis of IF-specific messenger RNA in the macrophage cultures. The present results suggest the existence of "preformed-IF" and "synthesized-IF" in the cultured mouse spleen cells *in vitro*. It is supposed that the two IF are the early- and late-IF *in vitro* by ET, respectively. It is well known that actinomycin D inhibits the synthesis of DNA dependent cell RNA, including messenger RNA (22) and puromycin efficiently suppresses protein synthesis in the cells (23). As described above, since the production of early-IF *in vitro* was not inhibited by actinomycin D, but was inhibited by puromycin, its preformed state may be suggested to be a long-life messenger RNA which always exists within the cells as an inactive precursor. Since the active form of IF appears to be a protein, an action of inducer such as ET may be an activation of translation mechanism for synthesizing IF-protein. Such a suggestion may be rather adventurous. However, recently, it was reported that the production of a 19S antibody was not inhibited by actinomycin D (24). This finding may suggest somewhat the regulation of the RNA-level. Further investigations are needed to explain the production mechanism of early-IF.

Summary. Mouse spleen cells cultured *in vitro* produced at least two kinds of interferon (IF) in response to inoculation with *E. coli* endotoxin. The early-appearing IF showed its peak titer about 2 hr after the inoculation of inducer, while the late-appearing IF possessed its maximum titer at 6–8 hr. Their properties were generally similar to those of endotoxin-induced IF in mouse plasma or spleen *in vivo*. The production of endotoxin-induced IF in mouse plasma or spleen was in parallel with the appearance of

lysosomal enzymes, β-glucuronidase and acid phosphatase. In the case of cultured spleen cells *in vitro*, such parallelism was not observed at all. The production of early-IF *in vitro* was not affected by treatment with actinomycin D (10μg/ml), but inhibited almost completely by puromycin (50 μg/ml). Both inhibitors caused almost complete inhibition of the production of late-IF.

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