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Immune-Specific Production of Gamma Interferon in Human Lymphocyte Cultures in Response to Mumps Virus

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The production of interferon (IFN) was investigated in human peripheral lymphocyte cultures stimulated by mumps virus. IFN-α and IFN-γ were produced in lymphocyte cultures from immune donors. However, no IFN-γ was produced in lymphocyte cultures from nonimmune donors. IFN-α was produced by B lymphocytes, and IFN-γ was produced by T lymphocytes. An increase in the production of IFN-γ resulted from the mixture of autologous macrophages in T-lymphocyte cultures. The production of IFN-γ by T lymphocytes was found to depend on prior, possibly cellular, immunity.

Interferon (IFN) has been classified into three types, α, β, and γ (8). IFN-α and -β are known to be acid stable and can be induced by some viruses, polyinosine:polycytosine, and other inducers (7). IFN-γ is acid labile, is not neutralized by anti-IFN-α and anti-IFN-β serum (7), and can be induced by the stimulation of nonspecific mitogens (4, 24, 25) and specific antigens (2, 3, 5, 6, 17, 20, 22). Immune-specific IFN production was found by Green et al. (5) in response to stimulation by purified protein derivative. Youngner and Salvin (27) reported that this type of IFN was different from so-called classical IFN in acid stability and antigenicity. This type of IFN was called type II IFN. At present, type II IFN is called IFN-γ (8). In the case of specific viral antigens, IFN-γ was produced by stimulation with herpes simplex virus (6, 17, 22), cytomegalovirus (20), and vaccinia virus (3).

Valle et al. (22) showed that IFN-γ was produced by T cells stimulated by herpes simplex virus and that production was increased when both macrophages and lymphocytes were present.

Recently, Kato and Minagawa (11) reported that IFN-α, but not IFN-γ, was produced in human lymphocyte cultures from seropositive donors in response to mumps virus. Similar results were reported with influenza A virus (12) and varicella-zoster virus (9). These results seem to suggest that not all viruses induce IFN-γ in immune response.

In this report, IFN production in response to mumps virus was investigated in human lymphocyte cutlures, and results different from those of Kato and Minagawa (11) were obtained.

MATERIALS AND METHODS

Sources of lymphocytes. Donors consisted of healthy children who visited the pediatric outpatient clinic of Saiseikai Central Hospital, Tokyo, Japan, and healthy members of the medical staff.

Cell lines. Vero cells and FL cells were grown in Eagle minimum essential medium supplemented with 5% calf serum. Namalva cells were grown in RPMI 1640 medium supplemented with 8% calf serum.

Mumps virus. In this investigation, the Sasazaki strain of mumps virus was used. After passage 4 times in Vero cells, the culture fluid was harvested and centrifuged at 3,000 rpm for 30 min. The supernatant was stored at −70°C until use. This preparation contained about 1.6×10^7 50% tissue culture infective dose ($TCID_{50}$) per ml. For the preparation of UV-inactivated mumps virus, 2 ml of the stocked live mumps virus in a 45-mm plastic plate was irradiated with 10 W of UV light at a distance of 15 cm under agitation.

Separation of mononuclear cells. Mononuclear cells were separated from heparinized peripheral venous blood by the Ficoll-Hypaque gradient centrifugation method described by Perper et al. (16). Interface cells were washed with phosphate-buffered saline (PBS) and suspended in RPMI 1640 medium supplemented with 2% fetal calf serum (FCS).

Preparation of macrophages. Mononuclear cells were adjusted to 4×10^6 viable cells per ml, determined by exclusion of trypan blue dye in RPMI 1640 medium supplemented with 20% autologous serum. A 5-ml amount of cell suspension was incubated in each plastic plate (65 by 15 mm) at 37°C in a humidified atmosphere of 5% CO_2. After 72 h, nonadherent cells were removed, and adherent cells were washed with PBS and incubated in fresh medium. After 1 week, adherent cells were vigorously washed three times with PBS, detached by scraping with a sterile rubber stopper, and resuspended in RPMI 1640 medium supplemented with 2% FCS (18).

Separation of T and B lymphocytes by nylon wool column. A glass wool column and a nylon wool column were prepared by the method described by Maehara and Ho (13). Fresh blood was obtained. About 0.5×10^8 to 1.5×10^8 mononuclear cells in 2 ml of minimum essential medium supplemented with 5% FCS were poured onto the glass wool column at room tempera-

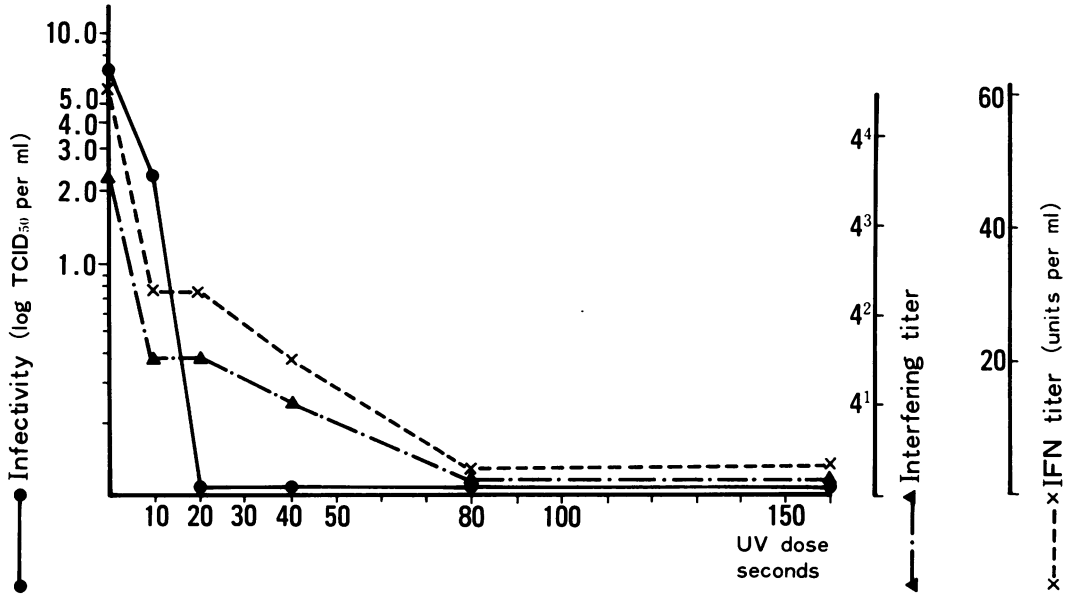

FIG. 1. Effects of UV irradiation on the biological activities of mumps virus. Symbols: ●, infectivity; ▲, interfering activity in FL cells against VSV; ×, IFN-inducing activity in Namalva cells.

ture. The effluent fraction, cleared of macrophages and dead cells, was centrifuged at 1,000 rpm for 10 min. Cells resuspended in 2 ml of warm medium were poured onto the nylon wool column, and this step was followed by adding 2 ml of warm medium. The column was incubated at 37°C for 45 min and then washed slowly with 30 ml of warm medium. For elution of nylon wool-adherent cells, the nylon wool was pressed and squeezed and then washed with 8 ml of warm medium. The cells obtained were centrifuged at 1,000 rpm at 4°C for 10 min and suspended in RPMI 1640 medium supplemented with 2% FCS. After this procedure, the effluent cells consisted largely of T cells and non-B–non-T cells, and the adherent cells consisted of B cells (10, 13, 21).

Separation of T and B lymphocytes by their interaction with SRBC. Mononuclear cells were adjusted to 4 × 10^6 per ml in RPMI 1640 medium supplemented with 2% FCS. Sheep erythrocytes (SRBC) were washed with PBS, treated with neuraminidase, and adjusted to a 1% suspension. Mononuclear cells were mixed with an equal volume of 1% SRBC, incubated at 37°C for 15 min, centrifuged at 1,000 rpm for 10 min, and incubated in an ice bath at 4°C for 2 h. The cell pellets were gently resuspended, carefully layered on Ficoll-Hypaque solution, and centrifuged at 1,600 rpm (400 × *g*) for 30 min. Interface cells were collected and washed with PBS and resuspended in RPMI 1640 medium supplemented with 2% FCS. Pellets were gently resuspended, mixed with 0.83% NH_4Cl at 4°C to lyse the SRBC, washed three times with PBS, and resuspended in RPMI 1640 medium supplemented with 2% FCS (26).

Induction of IFN. Lymphocytes of each fraction were incubated at a concentration of 2 × 10^6 per ml in RPMI 1640 medium supplemented with 2% FCS in a glass tube (13 by 80 mm) at 37°C and stimulated with 0.2 ml of live or UV-inactivated mumps virus. Every induction was done in duplicate or triplicate.

Treatment at pH 2. The lymphocyte culture supernatants were divided into two aliquots. One was untreated; the other was dialyzed with KCl-HCl buffer (pH 2) for 48 h at 4°C and then dialyzed against PBS for 48 h at 4°C. These paired specimens were stored at −70°C until the IFN assay.

IFN assay. IFN was assayed by a modified Armstrong semimicro dye binding method (14). Briefly, FL cells in a 96-well plate were incubated with 0.1 ml of IFN samples for 24 h at 37°C in a humidified atmosphere of 5% CO_2. The plate was then washed and inoculated with 0.1 ml of vesicular stomatitis virus (VSV) containing $10^{3.5}$ $TCID_{50}$ in each well. After incubation at 37°C for 17 h, the cultures were washed with physiological saline solution and stained with gentian violet. The IFN titer was expressed as the reciprocal of the highest dilution that showed 50% inhibition of the cytopathic effect of VSV compared with the reference human leukocyte IFN (G-023-901-527), supplied by G. Galasso, National Institutes of Health, Bethesda, Md.

Neutralization of IFN. Diluted IFN samples were mixed with an equal volume of a 1:50 dilution of anti-leukocyte IFN serum (DAB-0006), kindly supplied by G. Galasso. The serum had an antibody titer of 1:10,000 against human leukocyte IFN and 1:240 against human fibroblast IFN, expressed by the end-point dilution which neutralized 10 U of IFN. After incubation at 37°C for 60 min, the mixture was assayed by the same method used for the IFN assay.

NT to mumps virus. The neutralizing titer (NT) to mumps virus was assayed with 50% cytopathic effect inhibition in Vero cells. NT was expressed as the reciprocal of the serum dilution that produced 50% inhibition of cytopathic effect (19).

TABLE 1. IFN production in human blood lymphocyte cultures stimulated with live or inactivated mumps virus

Donor	Age	Mumps infection	NT	Cells/ml ($\times 10^6$)	Antigen (ml)	IFN (U/ml)	
						Not treated	Treated at pH 2
1	30 yr	22 yr before	45	2	0.1[a]	384	144
					0.1[b]	96	24
2	11 yr	Not clarified	16	2	0.1[b]	12	5
					0.2[b]	19	9
					0.3[b]	42	31
3	9 yr	Not clarified	36	2	0.1[b]	8	—[c]
					0.3[b]	24	14
4	1 yr	No	<4	2	0.1[a]	46	19
					0.1[b]	—	—
				2	0.1[a]	16	12
				6	0.1[a]	24	24
5	7 mo	No	8	2	0.1[b]	—	—
					0.3[b]	9	6
				6	0.3[b]	9	9

[a] Live mumps virus.
[b] Mumps virus UV irradiated for 80 s.
[c] —, Not detectable.

RESULTS

Effects of UV irradiation on the biological activities of mumps virus. The effect of UV irradiation on infectivity, IFN-inducing activity, and interfering activity with VSV are shown in Fig. 1. Live mumps virus contained 1.6×10^7 $TCID_{50}$ per ml, but infectivity was lost after 20 s of UV irradiation. To investigate the effect of UV irradiation on IFN-inducing activity, Namalva cells were adjusted to 10^6 per ml and stimulated with 0.1 ml of UV-irradiated mumps virus. After 3 days, culture supernatants were harvested, treated at pH 2, and assayed for IFN. Live mumps virus induced 60 U of IFN, and after 80 s of UV irradiation, all IFN-inducing activity was lost in Namalva cells.

If any mumps virus remained in the lymphocyte culture supernatants, the interference of mumps virus with VSV might have given a false IFN titer. Consequently, the interfering activity was examined as follows. Serial fourfold dilutions of mumps virus were placed on FL cells and followed by the same method as the IFN assay. Live mumps virus interfered with the cytopathic effect of VSV in FL cells, even in a >128-fold dilution. However, mumps virus irradiated by UV for 80 s had no interfering activity.

Therefore, an 80-s dose of UV irradiation was used in subsequent experiments to inactivate mumps virus.

IFN production in human lymphocyte cultures stimulated with live or UV-inactivated mumps virus. Human lymphocytes from seronegative and seropositive donors were stimulated by live or UV-inactivated mumps virus. The results are shown in Table 1. Donor 1 had had a clinically apparent mumps infection 22 years before and had an NT of 45 against mumps virus. A 384-U amount of IFN was induced in lymphocyte cultures by stimulation with 0.1 ml of live mumps virus. IFN decreased to 144 U after treatment at pH 2. Otherwise, 96 U of IFN was induced by 0.1 ml of UV-inactivated mumps virus, decreasing to 24 U after treatment at pH 2. Acid-labile IFN was produced in lymphocyte cultures from seropositive donors in response to stimulation with live or UV-inactivated mumps virus. However, lymphocytes from nonimmune donors did not produce detectable acid-labile IFN in response to stimulation with UV-inactivated mumps virus.

Kinetics of IFN production. Two immune donors were investigated, and the results are shown in Fig. 2. Lymphocytes were stimulated with 0.2 ml of UV-inactivated mumps virus by the method described above. Peak IFN production was reached on day 5 or 7. Acid-labile IFN was produced within 24 h, and an increase of acid-labile IFN production was observed between 3 and 5 days in donor 7 but not in donor 6.

Cellular sources of IFN. IFN production in T-cell and B-cell fractions separated by SRBC rosette formation are shown in Table 2. Donor 9 was 8 years old and had had mumps meningitis 2 weeks before. In donors 8 and 10, acid-stable

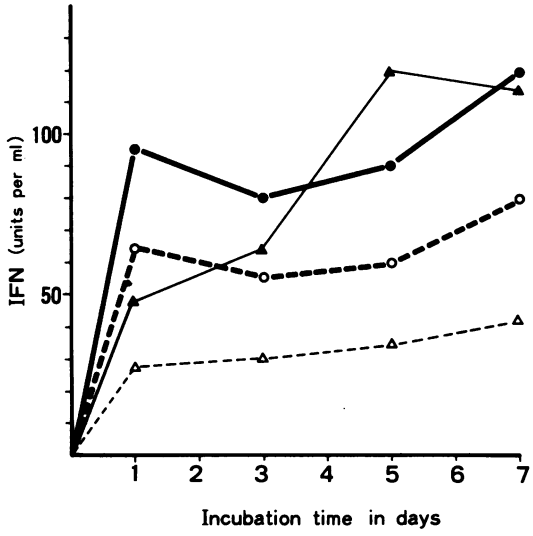

FIG. 2. Kinetics of IFN production in seropositive human blood lymphocyte cultures. Donor 6 was 24 years old and had an NT of 16 against mumps virus. The culture supernatant was treated (○) at pH 2 or not treated (●). Donor 7 was 9 years old and had an NT of 45 against mumps virus. The culture supernatant was treated (△) at pH 2 or not treated (▲).

IFN was produced by non-rosette-forming cells, but acid-labile IFN was not produced by macrophage-depleted rosette-forming cells. However, in donor 9, macrophage-depleted lymphocytes produced only acid-labile IFN. No IFN was produced by non-rosette-forming cells, and acid-labile IFN was produced by rosette-forming cells. In donor 10, autologous macrophages were mixed with lymphocytes of each fraction. Acid-labile IFN was produced by the admixture of macrophages in rosette-forming cells. Thus, acid-stable IFN was produced by non-rosette-forming cells, and acid-labile IFN was produced by rosette-forming cells.

Lymphocytes were also fractionated by a nylon wool column. IFN production in each cell fraction is shown in Table 3. Acid-stable IFN was produced by nylon wool-adherent cells, and acid-labile IFN was produced by nonadherent cells with or without the addition of macrophages. In donor 10, who contracted mumps about 20 years ago and has many contacts with children, acid-labile IFN was produced by nylon wool-nonadherent cells but not by macrophage-depleted rosette-forming cells (Table 2). It appears that the sensitivity of lymphocytes was decreased by fractionation by rosette formation. In donor 11, no acid-labile IFN was produced, even after autologous macrophages were added. In donor 12, acid-labile IFN was produced only by the mixture of macrophages with nonadherent cells.

These results show that acid-stable IFN is produced by B cells, acid-labile IFN is produced by T cells, and the mixture of autologous macrophages increases the production of acid-labile IFN by T cells in response to stimulation with UV-inactivated mumps virus.

Neutralization test of IFN. Rosette-forming cells from donor 9 were adjusted to 8×10^6 per ml and stimulated with 0.2 ml of UV-inactivated mumps virus (Table 2). A 128-U amount of IFN was induced; it was reduced to 16 U after treatment at pH 2. This IFN preparation was used for neutralization tests. The results are shown in Table 4. A 1:2.5 dilution revealed 32 U of IFN, and 16 U of IFN remained after treatment with anti-leukocyte IFN serum. The same amount of IFN-α was completely neutralized. A 40-U amount of IFN-β was neutralized by a 1:40 dilution of anti-leukocyte IFN serum (data not shown). These results reveal that nontreated IFN samples, including acid-labile IFN, contain IFN-γ.

DISCUSSION

IFN-γ is known to be induced by specific antigens (2, 3, 5, 6, 17, 20, 22) or nonspecific mitogens (4, 24, 25). Green et al. (5) reported immune-specific IFN production for the first time. They reported that lymphocytes from tuberculin-sensitive donors produced IFN-γ on exposure to purified protein derivative and that IFN production was maximum during days 4 to 7 of stimulation. Epstein et al. (2) also reported that the peak transformation for stimulation with purified protein derivative occurred on days 4 to 6, and peak IFN production occurred on days 7 to 8 after stimulation. The addition of macrophages to purified protein derivative-sensitized lymphocytes resulted in a more than threefold increase in IFN production.

Immune-specific IFN production in response to stimulation with herpes simplex virus was reported by Valle et al. (22) and by others (6, 17). Valle et al. (22) reported that IFN-γ production by combined macrophage-lymphocyte cultures was shown to depend on the presence of T cells. This IFN was relatively unstable at pH 2 and at 56°C, and the antigenicity was different from that of classical IFN (23).

In the case of stimulation with cytomegalovirus (20) and vaccinia virus (3), IFN-γ production was also reported.

Duc-Nguyen and Henle (1) reported that human peripheral lymphocytes supported, to some extent, the replication of mumps virus in the presence of phytohemagglutinin. McFarland et al. (15) reported that, in immune donors, a virus-specific proliferative response was elicited and occurred in T cells; more specifically, it oc-

TABLE 2. IFN production by T and B cells separated by SRBC rosette formation

Donor	NT	Cell fraction[a]	IFN (U/ml)	
			Not treated	Treated at pH 2
8	45	Mϕ-depleted lymphocytes	128	64
		R(−)	720[b]	643
		R(+)	—[d]	—
9	16	Mϕ-depleted lymphocytes	96	—
		R(−)	—	—
		R(+)	38	—
10	45	Mϕ-depleted lymphocytes	159	84
		R(−)	96	96
		R(+)	—	—
		Mϕ + Mϕ-depleted lymphocytes[c]	288	192
		Mϕ + R(−)	124	96
		Mϕ + R(+)	14	—

[a] Mϕ, Macrophages; R(−), non-rosette-forming cells; R(+), rosette-forming cells.
[b] —, Not detectable.
[c] Approximately 1.5×10^5 macrophages plus 2×10^6 lymphocytes.

curred in immunoglobulin G Fc receptor-bearing T cells (Tγ cells).

Kato and Minagawa (11) reported that IFN-α was produced in leukocyte cultures from seropositive donors, more precisely, by macrophages, non-B–non-T cells, and B cells, in response to stimulation with mumps virus. However, T cells did not produce any IFN, even in the presence of macrophages. The failure of IFN-γ production in lymphocyte cultures from immune donors was also reported in the case of stimulation with influenza A virus (12) and varicella-zoster virus (9).

Our results differ from those of Kato and Minagawa (11) on the point of IFN-γ production by T cells. INF-α was produced by B cells and IFN-γ was produced by T cells in response to UV-inactivated mumps virus. Macrophages stimulated IFN-γ production, but not all samples of lymphocytes from immune donors pro-

TABLE 3. IFN production by T and B cells separated by a nylon wool column

Donor	NT	Cell fraction[a]	IFN (U/ml)	
			Not treated	Treated at pH 2
10	45	Mϕ-depleted lymphocytes	159	84
		Adherent cells	30	30
		Nonadherent cells	12	—[b]
		Mϕ + Mϕ-depleted lymphocytes[c]	288	192
		Mϕ + adherent cells	120	60
		Mϕ + nonadherent cells	24	—
11	9	Mϕ-depleted lymphocytes	96	57
		Adherent cells	10	8
		Nonadherent cells	—	—
		Mϕ + Mϕ-depleted lymphocytes[c]	256	145
		Mϕ + adherent cells	16	16
		Mϕ + nonadherent cells	—	—
12	38	Mϕ-depleted lymphocytes	38	11
		Adherent cells	—	—
		Nonadherent cells	—	—
		Mϕ + Mϕ-depleted lymphocytes[c]	60	11
		Mϕ + adherent cells	18	10
		Mϕ + nonadherent cells	7	—

[a] Mϕ, Macrophages; adherent cells, nylon wool-adherent cells; nonadherent cells, nylon wool column effluent cells.
[b] —, Not detectable.
[c] Approximately 1.5×10^5 to 2.0×10^5 macrophages plus 2×10^6 lymphocytes.

TABLE 4. Neutralization of IFN by anti-leukocyte IFN serum

IFN sample	Antiserum treatment	IFN (U/ml)
IFN-α[a]	−	32
	+	—[b]
IFN[c] (donor 9)	−	32
	+	16

[a] IFN-α was produced in Namalva cell cultures stimulated with Newcastle disease virus.

[b] —, Not detectable.

[c] IFN (donor 9) was produced in rosette-forming cell cultures stimulated with UV-inactivated mumps virus.

duced IFN-γ. For example, the T cells obtained from donor 11, shown in Table 3, did not produce IFN-γ, even after the addition of macrophages. Lymphocytes from an individual who had had mumps 50 years before also did not produce IFN-γ, but after reimmunization with mumps vaccine, lymphocytes from the same individual produced a high titer of IFN-γ (data not shown). Significant amounts of IFN-γ were produced in donor 9, who was infected with mumps recently.

These results strongly suggest that the production of IFN-γ by T cells depends on the magnitude of immunity, possibly cellular immunity. This hypothesis may explain the difference of our results from those of Kato and Minagawa (11).

Our results agree with those of Valle et al. (22) and Rasmussen et al. (17). IFN-γ production is evaluated as a mediator of cell-mediated immunity. The migration of immune lymphocytes into the infected area and subsequent immune-specific IFN-γ production would play a significant role in host defense mechanisms.

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