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Mycoplasma Interaction with Lymphocytes and Phagocytes: Role of Hydrogen Peroxide Released from *M. pneumoniae*

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Interferon (IFN) production by human peripheral lymphocytes stimulated with *M. pneumoniae* was investigated. The hydrogen peroxide released from *M. pneumoniae* was responsible for the induction of IFN from lymphocytes, since horseradish peroxidase inhibited the IFN production and abrogated the activity of IFN production in the supernatant of *M. pneumoniae*. The antiserum neutralizing IFNα and IFNβ failed to neutralize partially interferon produced by lymphocytes. Treatment either with pH 2.0 or antiserum neutralizing human IFNγ resulted in a partial reduction of interferon. These results indicate that interferon produced by human lymphocytes stimulated with *M. pneumoniae* includes both types of IFNγ and IFNβ.

INTRODUCTION

Increasing evidence indicates that the production of different types of interferon (IFN) such as α, β, and γ are induced in immunocompetent cells as well as in other animal cells by various stimulators including virus, bacteria, synthetic polyamines, low-molecular synthetics, and mitogens [1]. Among human mycoplasmas, *M. pneumoniae* is considered a pathogen which causes upper respiratory infections and pneumonia in man. Also, *M. pneumoniae* produces hydrogen peroxide as a final metabolite of glucose which lyses erythrocytes of various animals [2,3]. On the other hand, there have been several reports that mycoplasmas [4,5,6,7,8] and mycoplasmatales virus [9] induced IFN production when inoculated in leukocytes and tissue-culture cells.

As we recently found that hydrogen peroxide induced IFN by peripheral lymphocytes, the present study was designed to determine whether the hydrogen peroxide produced by *M. pneumoniae* could induce IFN by *M. pneumoniae*-infected lymphocytes.

MATERIALS AND METHODS

Mycoplasma

Mycoplasma pneumoniae FH strain was supplied by Prof. M. Nakamura, Kurume University, Kurume, Japan. The microorganisms were cultured in PPLO liquid medium, without thallium acetate, at 37°C. After four days' cultivation, the culture was harvested and frozen at -80°C until use.

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Lymphocytes Preparation

Peripheral blood mononuclear cells were prepared from the heparinized peripheral venous blood of healthy adults by centrifugation over a Ficoll-Isopaque gradient [10]. The cells, designated peripheral blood mononuclear cells, were washed three times with Hanks' balanced salt solution (G-HBSS, Nissui Seiyaku Co., Japan) with 0.1 percent gelatin (Difco Laboratories, Detroit, MI). The residual erythrocytes were lysed by the addition of 1 ml cold Tris-0.83 percent NH_4Cl solution to the cell pellets in the tubes, followed by an incubation at 37°C for five minutes; the cells were then washed three times and resuspended in culture medium.

Purification of T Cells

Nonphagocytic nonadherent and T cell-enriched mononuclear cells were obtained from the peripheral blood treated with iron phagocytosis followed by Ficoll-Isopaque gradient centrifugation and nylon filtration as described [11]. The carbonyl iron-treated peripheral blood was centrifuged over a Ficoll-Isopaque gradient to separate mononuclear cells from the blood. The separated mononuclear cells were washed two times with G-HBSS and resuspended with culture medium containing 10 percent fetal calf serum (FCS, Grand Island Biological Co., Gibco, Grand Island, NY). The cell suspensions were then applied on a nylon wool column in the plastic syringe to remove adherent cells (B cells) [11]. These combined methods of iron phagocytosis and nylon filtration generally yield lymphocyte preparations containing less then 0.1 percent of either monocytes or B cells as determined by E rosette and myeloperoxidase staining [12].

Purification of B Cells

Mononuclear cells obtained from the carbonyl iron-treated peripheral blood by Ficoll-Isopaque density gradient centrifugatons were added in a ratio of 1:50 to sheep red blood cells (SRBC) treated with neuraminidase (from *Vibrio cholerae*, Behring-Werke AG, Marburg, West Germany) as described [11]. The mixture was incubated at 37°C for 10 minutes, spun down at 160 *g* for five minutes and then kept at 0°C for 45 minutes. After incubation, the mixture was resuspended very gently by pipetting. The cell suspensions were overlaid on Ficoll-Isopaque gradient and centrifuged at 150 *g* for five minutes and subsequently at 600 *g* for 10 minutes. The bound cells in the interface of the gradient were used as B cells. This procedure generally yielded lymphocyte preparations which contained more than 85–95 percent of B cells, the others being T cells.

Determination of the Number of Mycoplasmas

Ten microliters of each dilution were plated out on agar media in triplicate and incubated for six days at 37°C in a humidified chamber. The number of colony-forming units per milliliter was then determined by counting colonies on the agar plates.

IFN Assay

The antiviral activity was assayed by using 50 percent plaque reduction technique in WISH cells with vesicular stomatitis virus (VSV) as a challenge virus [13]. For the assay, 0.1 ml of the test materials diluted to a ratio of 1:2 was added in triplicate to monolayers of WISH cells in a 96-well plate (Nunc) and the plate was incubated at 37°C for 24 hours. After removing the sample solution, 30–40 plaque-forming units

(PFU) of VSV were added to each well and the plates were incubated at 37°C for 90 minutes to allow virus adsorption. After removing the unadsorbed virus, 0.1 ml of the medium containing 0.4 percent methyl cellulose (M281, Fisher Scientific Co., Fair Lawn, NJ) was added to each well. After incubation at 37°C for 24 hours, the medium was removed and the cells were stained with 1 percent crystal violet in ethanol to reveal virus plaques. IFN titers were expressed as reciprocal values of the dilution which reduced the number of virus plaques by 50 percent. A laboratory standard IFN preparation was included with each assay.

Culture Condition

Culture for cells was carried out in round-bottom microtest plates (Nunc Co. Ltd., Denmark). Each well contained 20×10^4 cells in 0.2 ml of RPMI 1640 (Gibco) fortified with 10 percent FCS and 2 mM L-glutamine. Cultures were then incubated in a humidified atmosphere of 5 percent CO_2 in air at 37°C. The supernatants were harvested and stocked at −80°C, until their antiviral activities were assayed.

Typing of IFN

Both antisera neutralizing human IFNα and IFNγ were purchased from Interferon Sciences Inc., New Brunswick, NJ. Antiserum to IFNα was a sheep antiserum to interferon produced in human leukocytes induced by Sendai virus. Antiserum to IFNβ was a rabbit antiserum to interferon produced in human fibroblasts induced by Sendai virus. Antiserum to IFNγ was a rabbit antiserum against purified IFNγ from plant lectin-stimulated human lymphocyte culture supernatants. IFNα and IFNγ used as controls were also purchased form Interferon Sciences Inc. For neutralization, 0.2 ml of test interferon preparations at 100 units/ml was mixed with an equal volume of antiserum or culture medium as control. After one hour of incubation at 37°C, residual interferon levels in the mixture were estimated.

RESULTS

Induction of IFN Production by M. pneumoniae *in Human Peripheral Lymphocytes*

Table 1 shows the results of experiments assessing the IFN production induced by *M. pneumoniae* in the unpurified peripheral mononuclear cells and their purified

TABLE 1
Induction of Interferon in Human Peripheral Mononuclear Cells by *M. pneumoniae* in Individuals

Donors	IFN Titers (units/ml)[a] PBL	T
1	279	558
2	209	508
3	488	1,097
4	200	2,650
5	210	533

[a] Lymphocytes (5×10^5/ml) were cultured with 10^6 CFU/ml of *M. pneumoniae* at 37°C for 72 hours. Antiviral activity in the supernatants was determined by the VSV-WISH plaque-reading method. The values of IFN represent the means of triplicate assays.

T-cell fractions isolated from different healthy donors. With all individuals tested, T-cell fractions (5×10^6/ml) produced 558 to 2,650 units/ml of IFN, when being incubated with 10^6 CFU/ml of *M. pneumoniae* for 72 hours, although the unpurified mononuclear cells, containing more than 70 percent of T cells, produced lower titers of IFN than that in purified T cells. Figure 1 shows the results of IFN production in culture fluid of unpurified mononuclear cells, purified T cells, B cells, and monocytes. IFN induced by 10^6 CFU/ml of *M. pneumoniae* in the T-cell fractions reached a maximum in about 12 to 24 hours. Figure 1 also shows that, even though using the optimal concentration of *M. pneumoniae* (10^6 CFU/ml), the titers of IFN were low in B cells or negative in monocytes throughout the observation period of three days.

The IFN production by T-cell fractions was induced by 10^6 to 10^4 CFU/ml of *M. pneumoniae,* which did not affect the viability of cells after a 24-hour cultivation.

Characterization of IFN

The types of IFN produced by *M. pneumoniae*-stimulated T cells were determined by antiserum neutralization and acid lability tests. As shown in Table 2, antiserum-neutralizing IFNα failed to neutralize significantly the IFN produced by T cells, under a condition that human fibroblast IFN induced by Sendai virus be completely neutralized. In contrast, the treatment with antiserum-neutralizing IFNγ resulted in a partial reduction of *M. pneumoniae*-induced but not fibroblast IFN. Treatment

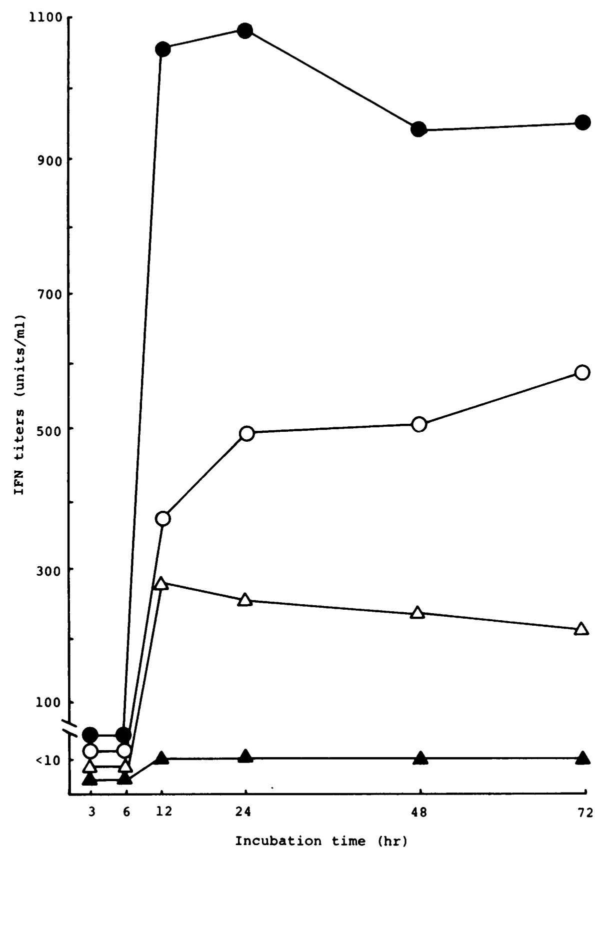

FIG. 1. Interferon titers by human peripheral T cells, B cells, and monocytes stimualted by *M. pneumoniae.* The suspension (0.1 ml) of *M. pneumoniae* (10^7 CFU/ml) was added to 10^6 cells in 1 ml of RPMI 1640 plus 10 percent FCS. Antiviral activities in each culture fluid after 72 hours' cultivation were measured by the plaque reduction method of WISH-VSV infection. (–○–), PBL; (–●–), T cells; (–△–), B cells; (–▲–), monocyte.

TABLE 2
Characterization of *M. pneumoniae*-Induced Interferon by Antibody Neutralization[a] and Low pH Stability Test[b]

Treatment	IFN	Residual Interferon Activity (% of control)
Anti-IFNα+β	Mycoplasma IFN	65
	HF IFN	10
Anti-IFNγ	Mycoplasma IFN	40
	HF IFN	98
pH 2.0	Mycoplasma IFN	56
	HF IFN	92

[a] Results are those from a representative experiment out of three experiments with similar results. Mycoplasma IFN: interferon produced by T cells; HF IFN: partially purified human IFNα induced by Sendai virus.
[b] Samples were dialyzed against Sörensen's glycine-HCl buffer [pH 2.0] for 24 hours at 4°C. The samples were returned to pH 7.4 by dialysis against phosphate-buffered saline [pH 7.4] prior assay.

with pH 2.0 also partially inactivated mycoplasma IFN but not fibroblast IFN. Therefore by both criteria a part of the IFN induced by *M. pneumoniae* was IFNγ.

Abrogation of IFN Production with Horseradish Hydrogen Peroxidase

As we have recently observed that hydrogen peroxide induced IFN production by human lymphocytes [14], we examined whether hydrogen peroxide released from *M. pneumoniae* stimulated IFN production. Graded numbers (CFU/ml) of *M. pneumoniae* were inoculated to 5×10^5/ml of T cells with or without 100 mcg/ml of hydrogen peroxidase, and then IFN titers in the supernatant of cell cultures after 72 hours' cultivation were assayed. As shown in Table 3, horseradish hydrogen peroxidase markedly inhibited IFN production by T cells stimulated by *M.*

TABLE 3
Inhibition of Horseradish Peroxidase on IFN Production by *M. pneumoniae*-Infected Human Peripheral Lymphocytes[a]

M. pneumoniae (CFU/ml)	Peroxidase	IFN Titers[b] (units/ml)
10^6	−	990
	+	160
10^5	−	533
	+	37
10^4	−	560
	+	55
10^3	−	20
	+	20

[a] T cells (10^6/ml) were incubated in presence or absence of 100 mcg/ml of hydrogen peroxidase with graded number CFU of *M. pneumoniae.*
[b] The values of IFN represent the means of triplicate assays.

TABLE 4
Effects of Peroxidase Addition Times on
IFN Production Induced by *M. pneumoniae*

Addition Time (hours) of Peroxidase (10 mcg/ml) After Cultivation[a]	IFN Titers[b] (units/ml)
0	40
3	50
6	39
16	101
24	209

[a] *M. pneumoniae* (10^5 CFU/ml) was inoculated in 0.2 ml of T-cell suspensions (1×10^6/ml). Data represent a representative experiment of three experiments with similar results.
[b] IFN titers in supernatants of T cells were measured after 24 hours' cultivation. The values represent the means of triplicate assays.

pneumoniae. In an additional experiment, 10 mcg/ml of peroxidase was added to the cultures of mycoplasma-infected lymphocytes at intervals during cultivation. The inhibition of IFN production by peroxidase was clearly observed when peroxidase was added after six hours of cultivation (Table 4). The addition of peroxidase, however, after 18 hours of cultivation showed only weak inhibition of IFN production.

Next, we examined whether the hydrogen peroxide released from *M. pneumoniae* directly induced IFN production by lymphocytes. One hundred milliliters of *M. pneumoniae*-culture fluid (10^6 CFU/ml) were centrifuged at 2×10^4 *g* for 30 minutes, and suspended in 10 ml of RPMI 1640 tissue culture medium. After the suspensions had been incubated at 37°C for two hours, 1 ml of the supernatant obtained by centrifugation was added to 1 ml of T cells (5×10^6/ml) with or without hydrogen peroxidase. After three days of incubation, IFN titers in supernatants of T cells were assayed. As shown in Table 5, the additions of hydrogen peroxidase to the supernatant markedly inhibited the production of IFN; also hydrogen peroxide (12.5 mcg/ml) in the supernatant of *M. pneumoniae* suspension was reduced from 10^{-2} mM to less then 10^{-4} mM measured by the colorimetric method [15].

These results suggest that IFN production by lymphocytes stimulated by *M. pneumoniae* are, at least in part, due to hydrogen peroxide released from *M. pneumoniae*.

TABLE 5
Abrogation of IFN Produced by T Cells Stimulated with
the Supernatants of *M. pneumoniae* by Peroxidase[a]

Concentration of Peroxidase (mcg/ml)	IFN Titers (units/ml)[b] (% of reduction)
25	26 (68)
12	38 (53)
6	80 (0)
–	80

[a] Data represent a representative experiment out of three experiments with similar results.
[b] IFN titers in supernatants of T cells (1×10^6/ml) were measured after 72 hours' incubation at 37°C.

DISCUSSION

There have been several reports that mycoplasmas induced IFN production by human and animal leukocytes and by tissue-culture cells. Cole et al. reported that some mycoplasmas, such as *M. pneumoniae, M. gallisepticum,* and *Acholeplasma laidlawii* that produced hydrogen peroxide as a final metabolite of glucose, induced IFN production by ovine leukocytes [4,5,6], though *M. orale* 1 and *M. synoviae* also induce IFN production without glucose metabolites by tissue-culture cells and leukocytes, respectively [7,4]. However, the components of each mycoplasma as IFN inducers are not clear. In the present study we demonstrated that hydrogen peroxide released from *M. pneumoniae* could induce IFN production by human peripheral lymphocytes. The IFN was also composed, at least in part, of IFNγ.

Although IFNγ was considered to be produced in T cells stimulated by specific antigen, lectins [1], the production of IFNγ accompanying T-cell activation, has been observed after oxidation of the cell surface by galactose oxidase [16,17]. This enzyme produces aldehydes at the cell surface which cause the cross-link with amino groups [18], initiating the IFN production of T lymphocytes through the formation of Schiff bases [17,19]. On the other hand, oxidative metabolites, hydrogen peroxide, superoxide anion, and hydroxyl radical produced cytotoxic metabolites such as malonaldehyde in the cell membrane [20,21]. These substances were also capable of interacting with DNA and producing mutations in cross-linking with amino groups of DNA through the formation of Schiff bases [22,23]. These reports, that hydrogen peroxide released from *M. pneumoniae* as a final metabolite of glucose, may show a similar effect at the surface of T cells. Ten mcg/ml of peroxidase clearly blocked IFN induction, when added after three to six hours of cultivation, but not after 18 hours. This suggested that the contact of peroxide for certain periods was required for induction of IFN by lymphocytes. It may be important to consider an immunological circuit mediated with peroxide which involves some mycoplasmas and activated macrophages, since IFN augments the activity of natural killer cells and regulates antibody production and cellular immunity.

REFERENCES

1. Stewart WE II: Interferon inducers. In The Interferon system. Edited by WE Stewart II. Wien, New York, Springer-Verlag, 1979, pp 27–59
2. Cole BC, Ward JR, Martin CH: Hemolysin and peroxide activity of mycoplasma species. J Bacteriol 95:2022–2030, 1968
3. Somerson NL, Walls BE, Chanock RM: Hemolysin of Mycoplasma pneumoniae: Tentative identification as a peroxide. Science 150:226–228, 1967
4. Cole BC, Overall JC, Lombardi PS, et al: Induction of interferon in ovine and human lymphocyte cultures by mycoplasmas. Inf Immun 14:88–94, 1976
5. Rinaldo CR Jr, Cole BC, Overall JC, et al: Induction of interferon in mice by mycoplasmas. Inf Immun 10:1296–1302, 1974
6. Rinaldo CR Jr, Cole BC, Overall JC, et al: Induction of interferon in ovine leukocytes by species of mycoplasma and acholeplasma. Proc Soc Exp Biol Med 146:613–618, 1974
7. Rinaldo C, Overall JC, Cole BC, et al: Mycoplasma-associated induction of interferon in ovine leukocytes. Inf Immun 8:796–803, 1973
8. Birke C, Peter HH, Langenberg U, et al: Mycoplasma contamination in human tumor cell lines: effect on interferon induction and susceptibility to natural killing. J Immunol 127:94–98, 1981
9. Lombardi PS, Cole BC: Induction of a pH-stable interferon in sheep lymphocytes by mycoplasmatales virus MVL2. Inf Immun 20:209–214, 1978
10. Terukina S, Arai S: Activation of human T and non T lymphocytes by Sepharose-bound concanavalin A and the differential effect of macrophages. Immunology 44:215–222, 1981
11. Arai S, Yamamoto H, Itoh K, et al: Suppressive effect of human natural killer cells on pokeweed mitogen-induced B cell differentiation. J Immunol 131:1–7, 1983

12. Kaplow PE: Substitute for benzidine in myeloperoxidase stains. Amer J Clin Pathol 63:451–452, 1975
13. Handa K, Suzuki H, Matsui H, et al: Natural killer (NK) cells as a responder to interleukin 2 (IL-2) II. IL-2-induced interferonγ production. J Immunol 130:988–992, 1983
14. Arai S, Munakata T, Kuwano K: Induction of interferon and augmentation of the NK activity in human mononuclear cells by hydrogen peroxide. Excerpta Medica, in press
15. Pick E, Keisari Y: A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol 38:161–170, 1980
16. Dianzani F, Monahan TM, Scupham A, et al: Enzymatic induction of interferon production by galactose oxidase treatment of human lymphoid cells. Inf Immun 26:879–882, 1979
17. Dixon GFP, Parker JW, O'Brien RL: Transformation of human peripheral lymphocytes by galactose oxidase. J Immunol 116:575–578, 1976
18. Novogrodsky A, Katchalski E: Induction of lymphocyte transformation by sequential treatment with neuraminidase and galactose oxidase. Proc Nat Acad Sci USA 70:1824–1827, 1973
19. Greineder DK, Rosenthal AS: The requirement for macrophage-lymphocyte interaction in T lymphocyte proliferation induced by generation of aldehydes on cell membranes. J Immunol 115: 932–936, 1975
20. Stossel TP, Mason RJ, Smith AL: Lipid peroxidation by human blood phagocytes. J Clin Invest 54: 638–644, 1974
21. Shamberger KJ: Increase of peroxidation in carcinogenesis, J Nat Canc Inst 48:1491–1493, 1972
22. Reiss V, Tappel AL, Chio KS: DNA-malonaldehyde reaction: formation of fluoresecent products. Biochem Bioph Res Comm 48:921–926, 1972
23. Mukai FH, Goldstein BD: Mutagenicity of malonaldehyde, a decomposition product of peroxidized polyunsaturated fatty acids. Science 191:868–869, 1976