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Membrane interactions involved in the induction of interferon-alpha by *Mycoplasma pneumoniae*

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Summary

Interferon (IFN) induction by *Mycoplasma pneumoniae* (MP) in human peripheral blood mononuclear cells (PBMC) has been studied. We show that IFN yields depend on the concentration of both lymphocytes and MP. The effective IFN inducer appears to be the mycoplasma membrane, and IFN production occurs without significant lymphocyte proliferation. Data obtained by both positive and negative selection experiments suggest that the PBMC subpopulation induced by MP is B lymphocyte, as opposed to the main subpopulation induced by NDV that is monocyte. Evidence is also provided suggesting that the membrane interaction between MP and the B cells is mediated by MHC Class II antigens.

Interferon; *Mycoplasma*; cell membrane; MHC Class II antigens

Introduction

Viral RNA, either single- or double-stranded, appears to be the critical factor in triggering induction of interferon-(IFN) beta (Field et al., 1967; Billiau et al., 1970; Dianzani et al., 1974). In contrast, in the case of viral induction of IFN-alpha, there is evidence that envelopes, rather than nucleic acids, are the critical factor (Dianzani et al., 1980). It has also been demonstrated that tumor cells and virus-infected cells are capable of inducing IFN-alpha (Svet Moldavski et al., 1974;

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Trinchieri et al., 1978; Blalock et al., 1979; Lebon et al., 1982), suggesting that simple membrane contact of the inducers with leukocytes is sufficient to trigger the induction process. In addition, higher yields of IFN are obtained by using virus-infected cells as opposed to live or inactivated virions, suggesting that membrane interaction is more important than viral infection as a mechanism for induction of IFN-alpha in vivo (Capobianchi et al., 1985).

IFN-alpha production has been reported to occur after exposure of leukocytes to a number of microorganisms different from viruses (Baron et al., 1981–82), such as *Corynebacterium parvum* (Vilcek et al., 1980), and bacterial products, such as *Staphylococcus aureus* protein A and lipopolysaccharide (LPS) from several bacteria (Maehara and Ho, 1977). In some instances the induction of IFN-alpha by tumor cells could be attributed to contaminating *Mycoplasma* present in the inducing cell cultures (Beck et al., 1980). Mycoplasmas are small bacteria lacking cell wall components. Some species cause infections of the genito-urinary and respiratory tracts of humans (Razin, 1985) e.g., the atypic primary pneumonia, caused by *Mycoplasma pneumoniae* (MP). *Mycoplasmas* growing in vivo or in cell culture adhere tightly to cells. It has been proposed that the organisms possess receptors which recognize MHC class II antigens (Stanbridge et al., 1981).

In this paper we analyze the membrane interaction between human PBMC and MP leading to IFN-alpha induction. Furthermore, the lymphocyte subpopulation responsive to IFN induction by MP has been analyzed by both negative selection and cell depletion experiments.

Materials and Methods

Mycoplasma culture

A clinical isolate strain of MP (Brenciaglia et al., 1977), was grown on a glass surface as described (Beck et al., 1980). It was suspended, after extensive washing with PBS, at a final concentration of 20 μg protein/ml and stored at −70°C until use. The organisms were inactivated by heating at 56°C for 45 min. Control of viability showed that MP did not survive this treatment, while antigenic properties, assessed by complement fixation assay, and electrophoretic pattern of major protein components, assessed by SDS PAGE analysis, were maintained.

Mycoplasma subcellular fractions

MP crude preparations were suspended in distilled water and freeze-thawed repeatedly. Membranes were separated from cytosol by differential centrifugation, as described (Biberfeld et al., 1974). Both preparations were adjusted to 20 μg protein/ml, as crude MP preparations, and stored at −70°C until use. Complement fixation titers of membrane preparations were comparable to those of crude MP preparations.

Interferon induction

Human peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-

Hypaque gradient centrifugation of blood from healthy donors. The culture medium was RPMI 1640 (Flow Laboratories Inc.), supplemented with 10% fetal calf serum and gentamycin (50 μg/ml). Cell viability, determined by the dye exclusion method, was higher than 90% in all experiments. The cells were incubated with the indicated amount of the IFN inducer. Viral induction of IFN was performed as previously described (Dianzani et al., 1970). Supernatant fluids were collected at different times and assayed for IFN content on human WISH amnion cells by Sindbis virus hemagglutinin yield reduction, after a single growth cycle (Dianzani et al., 1979). IFN titers were standardized by using the NIH reference standard IFN (Ga 23-902-530), supplied by the National Institute of Allergy and Infectious Diseases. The titers are reported in International Units (IU). Results are the mean of at least 3 separate experiments. Blastogenic activity was determined by [^{3}H]thymidine incorporation as previously described (Dianzani et al., 1979).

Lymphomonocyte subpopulations

PBMC were subjected to repeated cycles of adherence on gelatin-coated plates. Adherent cells were repeatedly washed and detached by treatment with medium containing 3 mM EGTA as described (Chien et al., 1983). The recovered cells were resuspended in complete RPMI medium and consisted of 97–99% monocytes as determined by morphology and nonspecific esterase staining. Enriched T lymphocytes were separated from non-T cells by rosetting non-adherent cells with neuraminidase-treated sheep red blood cells (SRBC) as previously described (Parish et al., 1974). Only 2% contaminating monocytes were present in T-enriched cells, and 96% of these cells rerosetted with SRBC. The B lymphocyte-enriched cells contained 65–80% surface immunoglobulin-bearing lymphocytes, as assessed by direct immunofluorescence staining.

Monoclonal antibody treatment

10^7 PBMC were incubated with 200 μl of undiluted monoclonal antibodies (MoAb) anti-Leu7 and anti-DR (Beckton Dickinson) for 30 min at 4°C in a final volume of 1 ml, adjusted with RPMI medium. After 2 washes with medium, they were incubated in 1 ml of RPMI medium in the presence or absence of 20 μl of guinea pig complement (30 min at 37°C). Adequacy of antibody and complement concentrations were assessed by fluorescence microscopy, to effectively deplete the cells positively stained with the corresponding antibodies. Specifically, before treatment the cells positively stained with anti-Leu7 and anti-DR MoAb amounted to 20 and 21% respectively. These values dropped to 3 and 4%, respectively, after treatment with antibody and complement. After treatment, depleted PBMC were washed twice with medium and incubated with the inducers.

Results

Mycoplasma structures responsible for induction of IFN-alpha

Optimal conditions for induction of IFN-alpha were defined by incubating hu-

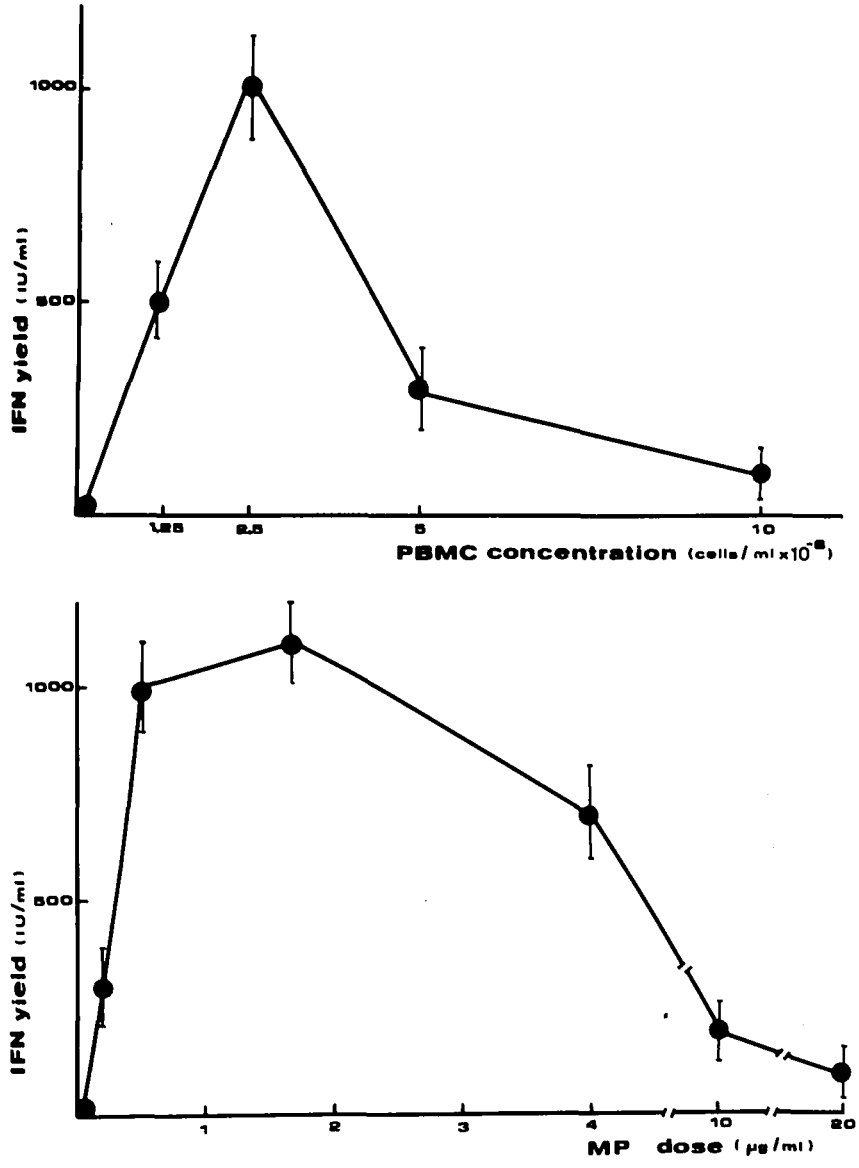

Fig. 1. 24 h IFN production by PBMC after stimulation with increasing doses of MP. Panel A: PBMC at the indicated concentrations were seeded in the presence of 1 µg/ml of MP. Panel B: PBMC were seeded at a density of 2.5×10^6 cells/ml in the presence of the indicated MP protein concentration. Bars indicate standard error of the mean over 3 independent experiments.

man PBMC at different concentrations with increasing does of MP for different times. Heat-killed MP were used in order to avoid effects and influences of active infection, or of MP metabolites, such as *Mycoplasma*-derived peroxide or mitogens. The results are shown in Fig. 1. It can be seen that MP induced up to 10^3 IU/ml of IFN, and that IFN yields depended on both MP concentration and lymphocyte density. Both curves are bell-shaped, with maximal IFN production occurring at lymphocyte density of 2.5×10^6/ml, and MP protein concentration of 1–3 μg/ml. Higher MP and PBMC concentrations were less effective. This finding is similar to that observed in induction of IFN-alpha by virus-infected cells. Therefore, in subsequent experiments PBMC and MP concentrations of 2.5×10^6 cells/ml and 2 μg/ml, respectively, were used. In exploratory experiments no increase of IFN titer was found after an incubation longer than 24 h until 7 days (data not shown); therefore supernatant fluids were collected for IFN determination after 24 h incubation. IFN produced in these experimental conditions was demonstrated to be of alpha type by acid stability and by neutralization with specific antiserum as previously described (Capobianchi et al., 1985).

To determine which MP component is responsible for IFN induction, we evaluated the ability of heat-killed whole MP as compared to that of sonicated MP, MP membrane preparations and internal MP components. The results of these experiments, reported in Table 1, suggest that the inducing activity in MP is attributable to membrane components, as they are sufficient to induce substantial amounts of IFN in PBMC cultures whereas internal MP material appears to be inactive. In fact, whole MP induced essentially the same amounts of IFN as the MP membranes, while cytosolic MP components were totally inactive.

To test whether IFN induction by MP parallels proliferation, blastogenesis was evaluated by [^{3}H]thymidine incorporation on day 5 after induction in the experi-

TABLE 1

IFN production and blastogenic response in cultures of human PBMC induced with Staphylococcal enterotoxin B (SEB), whole heat-killed *Mycoplasma pneumoniae* (MP), sonicated MP, MP membrane preparation, MP cytosol fraction

Induction schedule[a]		24 h IFN yield	Blastogenic response
Inducer	Dose (μg/ml)	(Log_{10} IU/ml)	([^{3}H]thymidine incorporation-cpm)
None	–	0.5	384 (± 102)
SEB	0.5	2.7 (± 0.1)[b]	9455 (± 1331)
Whole heat-killed MP	2	2.9 (± 0.1)[c]	348 (± 123)
Sonicated MP	2	2.9 (± 0.1)[c]	350 (± 85)
MP membranes	2	2.6 (± 0.1)[c]	367 (± 75)
MP cytosol	2	1.0	322 (± 69)

[a] PBMC suspensions of 2.5×10^6 cells/ml were incubated with indicated doses of inducers. Supernatants for IFN titration were harvested after 24 h. Blastogenic response was evaluated by [^{3}H]thymidine incorporation on day 5. Data are averages of 3 independent experiments, ± standard error of the mean.

[b] Characterized as IFN gamma.

[c] Characterized as IFN alpha.

mental conditions described above. A commonly used T-lymphocyte mitogen, Staphylococcal Enterotoxin B (SEB) was used as control for PBMC proliferation in the same experiments. Results, reported in Table 1, show that no lymphoproliferation is associated with the IFN alpha production after induction with MP, suggesting that the interaction of MP with PBMC is different from mitogenic stimulation.

PBMC subpopulation responsive for IFN production after MP induction

To investigate which PBMC subpopulation is induced by MP, we performed 2 types of experiments, based either on positive or on negative selection. Specifically, PBMC subpopulations were separated by glass adherence followed by E-rosetting separation. Three different populations were obtained, enriched in macrophages, T cells and non-T lymphocytes, respectively. These cells were incubated with MP as compared to a conventional IFN-alpha inducer, Newcastle disease virus (NDV). Results, shown in Fig. 2, suggest that the main responder cells to IFN induction by MP are non-T lymphocytes, whereas both monocytes and T lymphocytes are poor responders. In contrast, induction by NDV was most effective in monocyte-enriched cells, while some IFN production was found in non-T lympho-

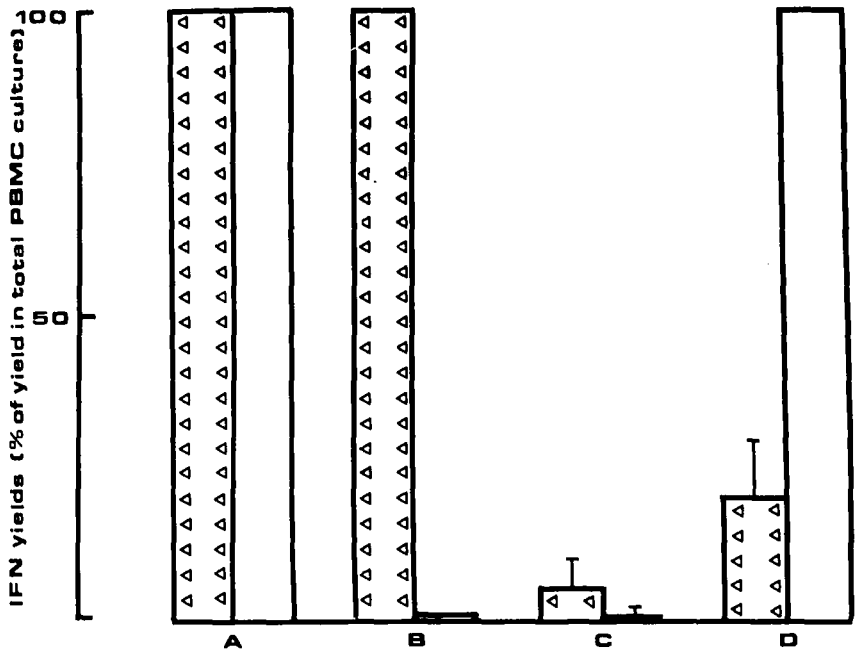

Fig. 2. 24 h IFN production by different PBMC subpopulations after stimulation with either NDV or MP. IFN titers are expressed as % of the production by total PBMC. Actual IFN titers in PBMC cultures stimulated by NDV and MP were 10,000 and 500 IU/ml, respectively. A = PBMC population; B = macrophage-enriched population; C = T cell-enriched population; D = B cell-enriched population. Bars indicate standard error of the mean over 3 independent experiments.

TABLE 2

Effect of pre-treatment with anti-Leu7 and anti-DR antibody, in the presence or absence of complement, on the production of IFN by PBMC after induction with MP

Pretreatment of PBMC[a]		IFN yield (Log_{10} IU/ml)
Antibody	Complement	
none	−	2.5 (± 0.1)[b]
none	+	2.5 (± 0.1)
anti-Leu7	−	2.5 (± 0.1)
anti-Leu7	+	2.5 (± 0.1)
anti-DR	−	1.9 (± 0.1)
anti-DR	+	1.8 (± 0.1)

[a] See section Materials and Methods.
[b] Average of 3 independent experiments, ± standard error of the mean.

cyte-enriched cells and no IFN production was observed in T lymphocyte-enriched cultures.

Because non-T cells separated by E-rosetting contain both B and NK lymphocytes, we performed negative selection experiments by incubating PBMC with MP after treatment with the monoclonal antibodies, anti-Leu7 and anti-MHC Class II DR antigens, in the presence of complement. Thus, the PBMC population was depleted of large granular lymphocytes (LGL), including NK, and B cells respectively. Also macrophages and activated T lymphocytes were partially depleted by anti DR plus complement treatment, but this could not influence the final IFN yield, because MP does not induce macrophages and T lymphocytes, as shown in Fig. 2. Results, reported in Table 2, show that depletion of Leu7-positive cells had no effect on IFN induction by MP, whereas depletion of DR-positive cells reduced IFN yields by more than 80%. Therefore the PBMC cells that are induced by MP are non-T, Leu7-negative, DR-positive lymphocytes.

Furthermore, data from Table 2 show that, while treatment of PBMC with anti-Leu7 antibody did not affect the yield of MP-induced IFN, irrespective of the combined treatment with complement, treatment with anti-DR antibody caused a similar reduction in IFN yield in the presence or absence of complement, suggesting that not only depletion of DR-positive cells, but also masking of DR antigen on their membranes sensibly reduces their responsiveness to MP.

Discussion

IFN induction by *Mycoplasma* in both murine and human PBMC has been previously reported. We reexplored this field in view of the emerging evidence that IFN-alpha induction is triggered by a membrane phenomenon. We used heat-killed MP, in order to avoid the effects of active infection, or MP metabolites, such as peroxide or MP-derived mitogens which have been reported to induce IFN-gamma in PBMC cultures (Arai et al., 1983; Kirchner et al., 1984). In this paper we show that IFN yields depend on both lymphocyte density and MP concentration. The

decreased IFN production at higher inducer or PBMC concentration could be due to a less effective interaction between inducer and responder cells due to steric hindrance. Experiments done with subcellular fractions of MP indicated that the membrane of MP is the effective IFN inducer, and that IFN production occurs without significant lymphocyte proliferation.

IFN-alpha production after induction with several agents has been attributed to monocytes, LGL or B lymphocytes (Dianzani and Capobianchi, 1987). In our experiments we could rule out the involvement of monocytes, LGL and T lymphocytes in the induction of IFN by MP. Data obtained by both positive and negative selection experiments indicate that the cells which are effectively induced are non-T lymphocytes possessing the Leu7 (−), DR (+)-phenotype. These characteristics are compatible with those of B lymphocytes.

Our data confirm that NDV preferentially induces IFN in monocytes, although some responsiveness was observed in B lymphocytes as well. The data do not help define whether the lower IFN production by NDV-stimulated B cells is due to contaminant monocytes (Saksela et al., 1984) or whether NDV, differently from Sendai virus, is capable of marginally inducing B cells (Weissman, 1986). The problem of cross-contamination of cell population does not pose itself in relation to the data obtained with MP as an inducer, since in this case the macrophage-enriched population failed to react at all.

The addition of anti DR antibody markedly reduced IFN yields whether or not complement was added, indicating that the presence of MHC Class II antigens on lymphocyte membrane is essential for IFN induction by MP. These data are in agreement with the hypothesis that these antigens act as *Mycoplasma* receptors on human cells (Stanbridge et al., 1981). Along the same line, Hughes et al. (1986) in their studies on IFN induction in murine lymphocytes by tumor cells, have found evidence that the critical interaction between the cells is also mediated by MHC Class II antigens on the B lymphocytes. On the basis of these findings we propose that many, if not all, molecules which interact with MHC Class II antigens on B lymphocytes are potential inducers of IFN-alpha in such cells. Viruses, however, seem to induce IFN-alpha by a different type of membrane interaction. Experiments are in progress to confirm this view.

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