IN VITRO EFFECTS OF AN IMMUNOSTIMULATING BACTERIAL LYSATE ON HUMAN LYMPHOCYTE FUNCTION

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MLBL is an oral immunostimulating vaccine consisting of bacterial standardized lysates obtained by mechanical lysis of different strains of Gram-positive and Gram-negative bacteria that can cause acute and chronic infections of the respiratory tract. Previous studies suggested a stimulating effect of MLBL both on humoral and cellular immune responses. In the present study, the in vitro effects of MLBL on human lymphocyte effector functions and its mechanisms of action were evaluated. The results show that the most remarkable effects of MLBL on the immune system are: i) activation of the IL-2 receptor (IL-2Rα) on different lymphocyte subsets (B, CD4+ T and CD8+ T cells) involved both in humoral and cellular immune responses; ii) induction of cytokine synthesis (IL-2, IL-10, IL-12, IFNγ) in the immune competent cells that induce and regulate immune responses; iii) generation of CD4+ and CD8+ effector T cells. Overall, these results suggest that the therapeutic effect of MLBL on acute and recurrent infections of the respiratory tract is related to its ability to activate the responses of different subsets of immune competent cells both for humoral and cellular immunity. Moreover, these effects can be induced either by direct immune cell activation or through the generation and activation of immune effector cells.

The mucosal surfaces represent the principal ports of entry for most exogenous agents including microrganisms. Mucosal tissues are defended by a local secretory immune system, the mucosal immune system, that may act in concert with the systemic immune system. The secretory immune system provides an integrated network linking mucosal sites and regulating trafficking of mucosally activated lymphocytes from induction to effector sites. The antigenic experience of one mucosal surface can be reflected in immune effector expression at a distant surface (1). Based on this concept of a "common mucosal immune system" (2-5), there is currently great interest in the possibility of developing mucosal vaccines.

Oral vaccines composed by lyophilized bacterial lysates have been largely used in the past to improve the efficiency of the immune response to the predominant pathogens of the respiratory tract in subjects with recurrent bacterial infections (6-13). The efficiency of a vaccine to induce a protective immunity is determined by its ability to stimulate the generation of activated cells that can become effector cells after a new encounter with the same infective agent. This generation is mediated by the production of regulatory cytokines from immune competent cells that exert a major role in the induction and modulation of acquired immune responses (4, 14-16).

It is well known that different patterns of cytokines are produced by different lymphocyte subsets and that cytokine production by CD4-positive (CD4+) and CD8-positive (CD8+) T cells usually exhibits a Type 1 or a Type 2 phenotype (17-21). Type 1 lymphocytes mostly

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produce interleukin-2 (IL2), interferon-γ (IFNγ) and tumor necrosis factor (TNF), are primarily involved in inflammatory cellular immunity and are essential for protection against a variety of intracellular infections. Type 2 lymphocytes produce other interleukins such as IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 and are responsible for the regulation of humoral immunity against certain extracellular infections. It is thought that the polarization towards Type 1 or Type 2 lymphocytes occurs at the first contact between the antigen and the immune competent cell. Effector cells are already generated after 4-5 days and their cytokine pattern is very close to the production pattern of related memory cells.

The development of mucosal and systemic immune response depends on the nature of antigenic stimulation and the eventual expression of Type1 versus Type 2 responses. Therefore, the study of the cytokine secretion pattern in the different subsets of immune competent cells activated by a vaccine represents an important information for evaluating its effectiveness and mechanism of action.

Bacterial extracts have been widely used as immunomodulators to prevent recurrent infections of the respiratory tract (6-13, 22-25). Several preparations containing bacterial lysates are licensed for use as vaccines. MLBL is a recently developed sub-lingual immunostimulating vaccine consisting of a standardized mechanical lysate (obtained through sonication) of 8 different bacterial strains of Gram-positive and Gram-negative bacteria (6 x 10^9 bacteria for each strain) selected among those most frequently implicated in acute and chronic infections of the respiratory tract (12, 13, 22). Inactivated micro-organisms or their sub-components, offer certain advantages as a potential vaccine for mucosal immunization. They are natural occurring microparticles, possess multiple antigens and are relatively inexpensive to produce. Previous studies suggested an immunopotentiating effect of MLBL on mucosal immune responses (22). The aim of the present work was to extend our knowledge on the effects of MLBL on the immune system and to study its mechanism of action. In particular, our study evaluated the *in vitro* effects of MLBL on IL-2R expression both in human B lymphocytes and in CD4+ and CD8+ T lymphocytes and on cytokine production both in naive or MLBL-sensitized human lymphocytes.

MATERIALS AND METHODS

Antigens

Lyophilized bacterial lysate obtained by mechanical lysis (ISMIGEN - MLBL, lot. N. 4488) was supplied by Zambon Italia SRL, Bresso/Milan, Italy. The lysate contained 6 x 10^9 bacteria for each of the following strains: *S. aureus, S. pyogenes, S. viridans, S. pneumoniae* (containing 10^9 bacteria of each of the following serotypes: TY1/EQ11, TY2/EQ22, TY3/EQ14, TY5/EQ15, TY8/EQ23, TY47/EQ24), *K. pneumoniae, K. ozaenae, H. influenzae serotype B, M. catharralis.* In brief, the method of preparation was the following: the bacteria were resuspended at the concentration of 1 x 10^{11} CFU/ml. The bacterial suspension was heat-inactivated, adjusted at pH 7.0, submitted to two homogenisation cycles at 630 bar by using an APV homogenisator (model 1000, Schenker) and lyophilised.

The experiments were performed by using the lyophilisate resuspended at the concentration of 5 mg/ml in RPMI 1640-GLUTAMAX (Gibco) culture medium, sonicated for 5 minutes. Dispersal was performed overnight at 4° C under slight agitation of the bacterial suspension. Then the bacterial suspension was aliquoted and stored at –80 °C until use.

PBMC separation and MLBL stimulation

Peripheral blood was obtained from healthy human donors. Mononuclear cells (PBMC) were isolated by Ficoll-hypaque gradient centrifugation. The mononuclear cells from the interface were harvested, washed and adjusted to 2 x 10^6/ml in RPMI1640-GLUTAMAX culture medium supplemented with 50 U/ml penicillin, 50 mg/ml streptomycin and 5% heath inactivated foetal calf serum (FCS).

Isolated PBMC were cultured alone or incubated with 10 and 100 mg/ml of MLBL in 6-well plates (Falcon) at 37 °C in humidified 5% CO_2 for 24 h. At the end of incubation, culture supernatants and the remaining cells were removed separately and used for successive evaluations.

Cytokine determination in culture supernatants

To determine extracellular cytokine concentrations, at the end of incubation time, cell cultures were harvested, centrifuged and the cell-free supernatants were collected and stored at –80 °C until assay.

Concentrations of IFNγ, IL-2, IL-10 and IL-12 (p40 subunit) in the culture supernatants were measured by two-site sandwich enzyme-linked immunosorbent assay (ELISA) using commercial reagents kits (Euroclone, UK), according to the protocol of the manufacturer. Cytokine levels were expressed as ng of protein per ml.

Detection of cell surface phenotype by two color immunofluorescence staining

At the end of incubation with MLBL, the cells were harvested, washed, resuspended in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 0.1% sodium azide (NaN_3) and were aliquoted for staining at 1 x 10^6 cells. To determine the cell surface phenotype for CD4, CD8 and CD19, respectively 50 µl of each of the following fluorochrome-conjugated monoclonal antibody (mAb) dilutions were used: CyChrome-conjugated mouse anti-human CD4 (PharMingen, San Diego, CA, USA), CyChrome-conjugated mouse anti-human CD8 (PharMingen), PE-conjugated mouse anti-human CD19 (Becton Dickinson, San Jose, CA, USA). Then, 50 µl of FITC-conjugated mouse anti-human CD25 (Becton Dickinson) were added and the cells were incubated for 30 min at 4 °C. As negative controls, aliquots of cell suspensions were incubated with an irrelevant isotype-matched mAb conjugated to the same fluorochrome as the sample. At the end of incubation the cells were washed twice and resuspended in PBS for flow cytometry.

Detection of cytokine-producing cells by intracellular immunofluorescence staining

To determine the frequency of cytokine-producing CD4+ and CD8+ T lymphocytes, the method described by Di Francesco et al. (26) with minor modifications was used. In brief, 5 hrs before harvest, 2 µM monensin (Sigma, St. Louis, MO) an inhibitor of intracellular transport, was added to block cytokine secretion in cultures (27). At the end of incubation, cultured cells were harvested, washed, resuspended in PBS containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3 and were aliquoted for staining at 1 x 10^6 cells. Then, 50 µl of FITC-conjugated mouse anti-human CD4 mAb (PharMingen), or FITC-conjugated mouse anti-human CD8 mAb (PharMingen) were added and the cells were incubated for 30 min at 4 °C. At the end of the incubation, the cells were washed and fixed in 2% paraformaldehyde for 20 min, then they were washed and resuspended in PBS containing 0.5% BSA, 0.01% NaN_3 and 0.1% saponin (Sigma, St. Louis, MO, USA) for permeabilization. For intracellular staining with anti-human cytokine mAbs, the following PE-labelled monoclonal antibodies, all purchased from PharMingen, were used: anti-human IL-2, anti-human IL-4, anti-human IL-10, anti-human IFNγ. As negative controls, aliquots of cell suspensions were incubated with an irrelevant isotype-matched mAb conjugated to the same fluorochrome as the sample. Finally, after the incubation at 4 °C for 30 min with the above mAbs, the cells were washed twice with PBS/BSA/NaN_3 without saponin and resuspended in PBS/BSA for flow cytometric analysis.

Flow cytometry

A FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 15 mW argon ion laser and filter setting for FITC (530 nm) and PE (585 nm) was used. Signals from light scatter channels and fluorescence-detecting photomultipliers (PMT) were collected in linear and logarithmic mode, respectively. The FL1 and FL2 channels were set up and compensated on a tight lymphocyte gate using tubes with a single-labelled strongly positive antibody. Dead cells were excluded by forward and side scatter gating. Ten thousand cells were recorded for each test and signals from all parameters were captured as list mode data using CellQuest software (Becton Dickinson). List mode files were analyzed for CD25 positivity or cytokine production by FACScan Research Software (Becton Dickinson) by first defining an analysis gate on anti-CD4, anti-CD8, or anti-CD19 positive cells. Irrelevant isotype-specific antibody was used as a control for non-specific binding of the anti-cytokine monoclonal antibodies. Statistical markers were set using the irrelevant isotype-matched controls as reference. Typically, 1% positive cells were allowed beyond the statistical markers in these controls. Previous results (28) showed that no significant cytokine immunofluorescence was present in fixed but not saponin-permeabilized cells. Data were expressed as the percentage of cells staining positive above the background staining.

Statistical analysis

Statistical significances of intergroup differences of normally distributed continuous variables was determined using Student's *t* test. P values of < 0.05 were considered statistically significant.

RESULTS

Effects of MLBL on cytokine concentrations in culture supernatants

Cytokine secretion was assessed by ELISA in the culture supernatants collected from the PBMC cultures treated or not with MLBL. The results (Fig. 1) showed that the incubation for 24 hrs of PBMC with MLBL at the dose level of 100 µg/µl induced the release in culture supernatants of significant amounts of all the tested cytokines, particularly IL-10 and IL-12. In control supernatants, IL-2 and IL-12 were completely absent (or their concentration was below the sensitivity limit of the test).

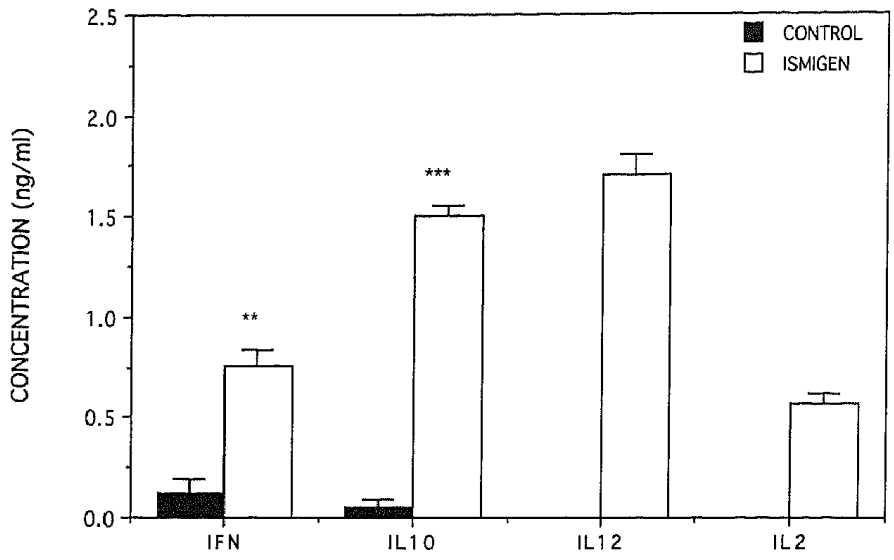

Fig. 1. *Isolated PBMC were incubated with 100 µg/µl of MLBL in 6-well plates and incubated at 37 °C for 24 h. At the end of incubation culture supernatants were harvested and analyzed by ELISA to determine IFNγ, IL10, IL12 and IL2 concentrations. Results are expressed as means ± S.E.M. of 6 independent experiments. Each experiment used PBMC from a different subject.*
*** P< 0.01*
**** P<0.001 (Student's t test).*

Effects of MLBL on CD25 (IL-2Ra) expression

The cells were incubated with MLBL for 24 h. At the end of this period the expression of CD25, the α chain of the IL-2 receptor, by the different cell subset was determined by two-color flow cytometry. The immunophenotypic analysis showed that incubation of PBMC with MLBL induced (Fig. 2) a significant increase of CD25 expression in both B and T lymphocytes. In T lymphocytes, MLBL treatment induces a statistically significant increase in the percentage of CD25+ cells in both CD4+ and CD8+ T subsets, when compared to naive cells (Fig. 2). In CD19+ B activated lymphocytes the frequency of cells expressing CD25 was significantly (P<0.01) higher respect to each of the T subset. No significant changes in the proportions of both CD4+ and CD8+ T cells were observed in either naive or stimulated cells after 24 hrs of MLBL treatment (data not shown).

Effects of MLBL on expression of intracellular cytokines

The production of cytokines by *in vitro* MLBL-stimulated immune CD4+ and CD8+ T cells was analyzed at the single cell level by simultaneous flow cytometric analysis of intracellular cytokines and surface antigen expression. PBMC were cultured for 24 hrs in the presence or absence of MLBL. At the end of incubation time, the frequencies of CD4+ or CD8+ T cells containing intracytoplasmic cytokines were determined analyzing, by two-color flow cytometric procedure, fixed and saponin-permeabilized cells fluorescent stained for the CD4 or CD8 surface molecules and for one of the intracytoplasmic cytokines IL-2, IL-4, IL-10 and IFNγ. As shown in Fig. 3, MLBL stimulation induced a statistically significant increase in the percentage of CD4+ T lymphocytes producing IL-2 and IL-10, whereas no significant effect was found in the same cells concerning IFNγ and IL-4 production. On the contrary (Fig. 4), in CD8+ T lymphocytes, there was, in addition to the increasing rate of IL-2 and IL-10-positive cells, also an increasing rate of IFNγ-producing cells. Likewise CD4+ T subset, also in CD8+ T cells the major increase was found in the percentage of IL-2-producing cells. No significant increase in the percentage of CD4+ and CD8+ T cells expressing the different types of cytokines was found after PBMC incubation with 10 µg/µl of MLBL (data not shown).

Effect of MLBL on cytokine production at the single-cell level after re-stimulation

To evaluate the effects of MLBL on intracellular cytokine production after re-stimulation, PBMC were incubated for 120 hrs with 100 µg/ml MLBL and then re-stimulated with a sub-optimal dose (10 µg/ml) of MLBL. The frequencies of CD4+ or CD8+ T lymphocytes singly expressing IL-2, IL-4, IL-10 and IFNγ were determined by two-color flow cytometric procedure. The results showed (Fig. 5 and 6) that the *in vitro* re-stimulation of PBMC with a sub-optimal dose of MLBL induced a marked increase versus unstimulated controls of the

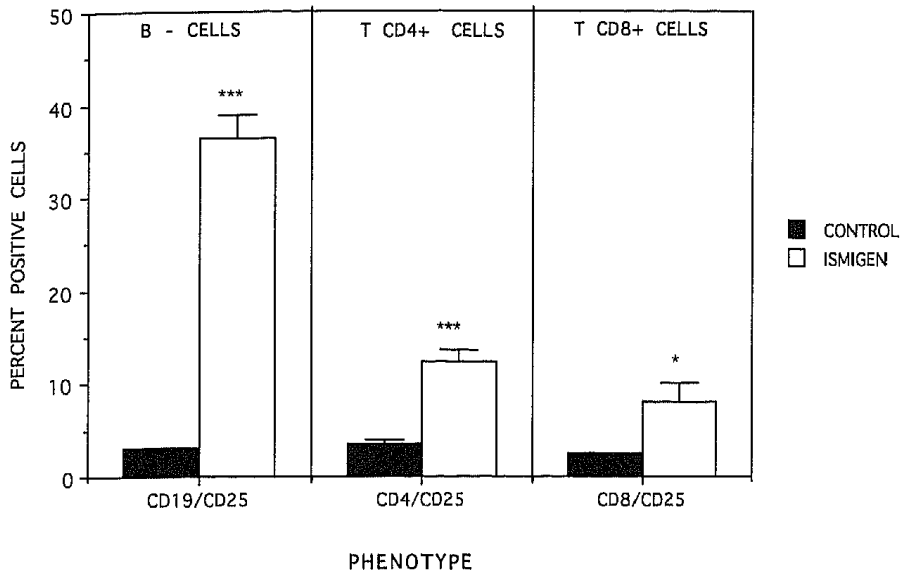

Fig. 2. *Effect of MLBL after 24 hrs of incubation on surface CD25 expression (the α chain of IL2 receptor) in CD19+ B cells and CD4+ and CD8+ T lymphocytes. The immunophenotypes were determined by FACS analysis and expressed as percentages of CD25+ cells in each lymphocyte subset. Results are expressed as means ± S.E.M. of 6 independent experiments. Each experiment used PBMC from a different subject.*
** P< 0.05*
****P<0.001 (Student's t test).*

number of individual CD4+ and CD8+ T subsets singly expressing IL-2, IL-4, IL-10 and IFNγ. In particular, the percentage of Type 1 (IL-2 and IFNγ) cytokine-producing cells was about the same in CD4+ and CD8+ T cell subsets, whereas the percentage of Type 2 (IL-4 and IL-10) cytokine-producing cells was statistically higher in CD4+ T subset. No significant changes in the proportions of both CD4+ and CD8+ T cells were observed either in naive or re-stimulated cells after MLBL treatment (data not shown).

DISCUSSION

The results of our experiments showed that the *in vitro* effects of MLBL on human immune cells mainly consist in: i) activation of the different naive lymphocyte subsets involved both in humoral and cellular immune responses; ii) induction of cytokine production by different immune regulatory cell subsets; iii) generation of antigen-experienced T cells showing an effector activity after re-stimulation with MLBL. Present findings confirm earlier experimental

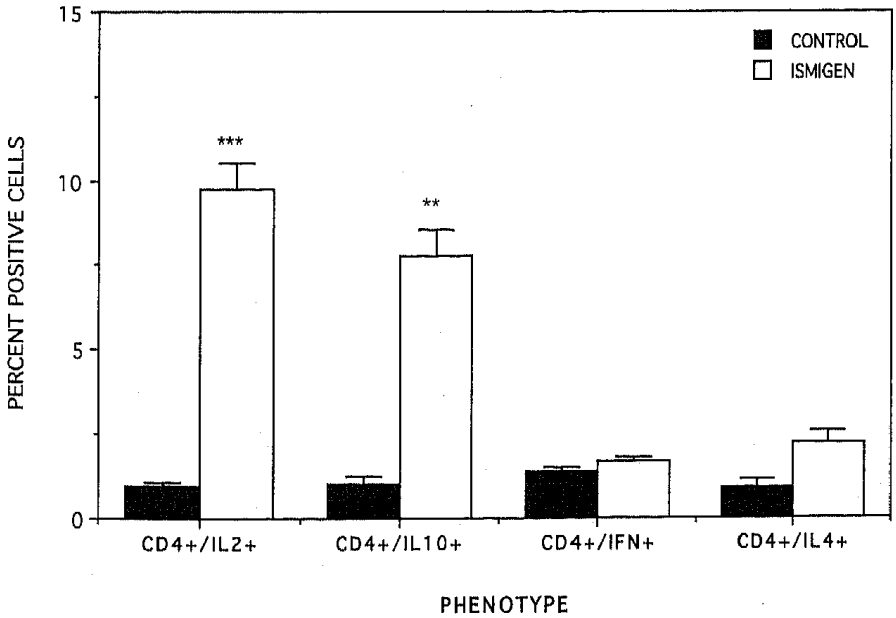

Fig. 3. *Effect of MLBL after 24 hrs of incubation on the frequency of CD4+ T lymphocytes producing IL2, IL10, IFNγ and IL4. All the samples were analyzed by two-colour flow cytometry for their surface CD4 antigen expression versus intracellular IL2, IL10, IFNγ and IL4 expression. Samples were analyzed by first gating on the CD4-positive population. The threshold for detection of positive cytokine-producing cells was set at 1% above the negative control consisting of cells treated with an irrelevant isotype-matched antibody. Results are expressed as means ± S.E.M. of 6 independent experiments. Each experiment used PBMC from a different subject.*

*** P< 0.01*
****P<0.001 (Student's t test).*

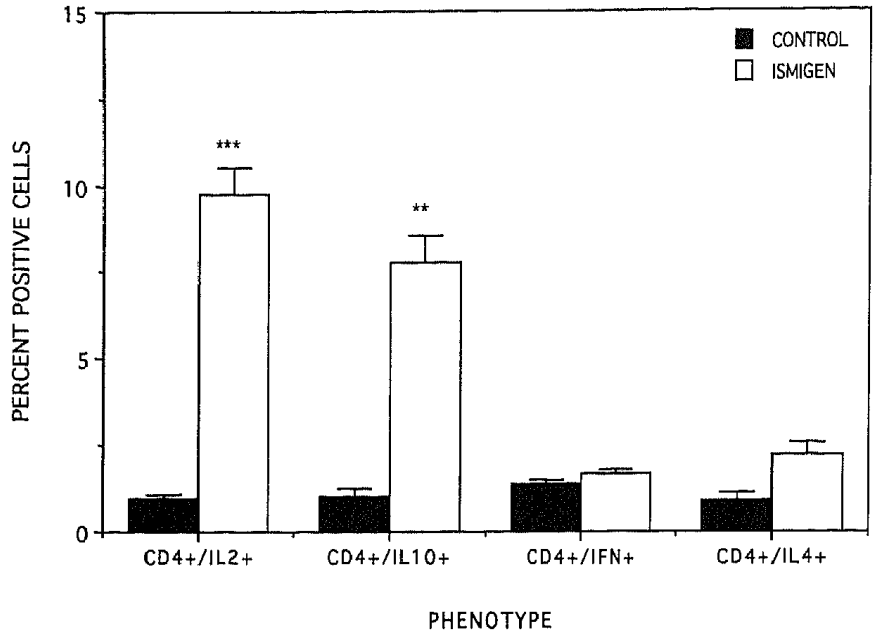

Fig. 4. *Effect of MLBL after 24 hrs of incubation on the frequency of CD8+ T lymphocytes producing IL2, IL10, IFNγ, and IL4. All the samples were analyzed by two-colour flow cytometry for their surface CD8 antigen expression versus intracellular IL2, IL10, IFNγ and IL4 expression. Samples were analyzed by first gating on the CD8-positive population. The threshold for detection of positive cytokine-producing cells was set at 1% above the negative control consisting of cells treated with an irrelevant isotype-matched antibody. Results are expressed as means ± S.E.M. of 6 independent experiments. Each experiment used PBMC from a different subject.*

** P< 0.05*
***P<0.01*
***P<0.001 (Student's t test).*

and clinical studies suggesting that MLBL can prime several immune responses against bacterial antigens, thus providing an effective barrier against invasion of pathogenic bacteria (5-13, 22-25).

In particular, the results demonstrated that MLBL treatment induced an increase in the percentage of either B lymphocytes or CD4+ and CD8+ T lymphocytes expressing CD25, a surface antigen corresponding to the α-chain of the IL-2 receptor (IL-2Rα) and considered to be an activation marker. This finding indicates that MLBL can modulate the IL-2-IL-2R signalling that is regarded as a pivotal mechanism in the development of immune response. In fact, the functional differentiation of lymphocyte subsets strictly depends on their activation by the Type 1 cytokine IL-2 (29) that exerts an essential role in the immune response by inducing lymphocyte

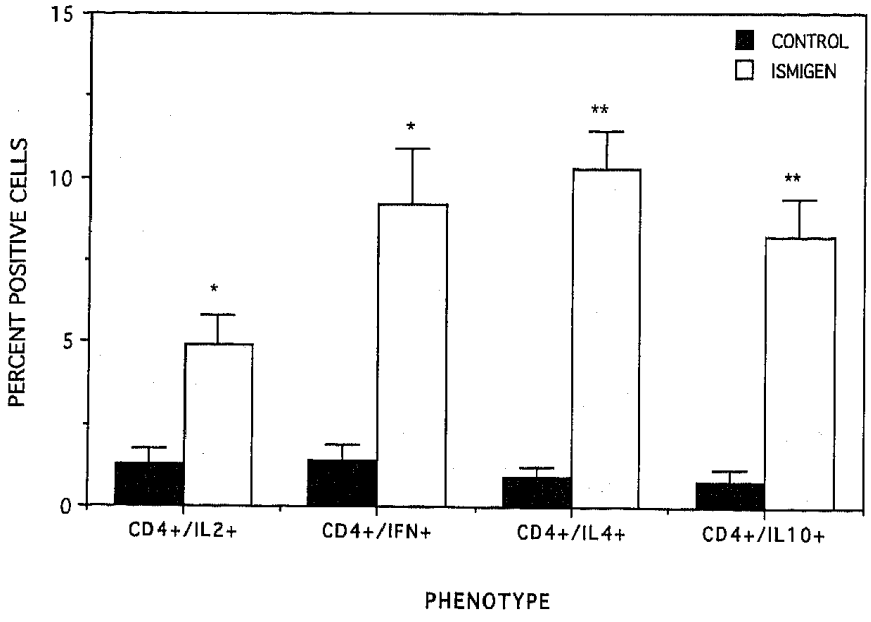

Fig. 5. *Effect of MLBL incubation on the frequency of CD4+ T lymphocytes producing IL2, IFNγ, IL4 and IL10 in re-stimulated cultures. PBMC were incubated for 120 hrs with 100 μg/ml MLBL and then re-stimulated with a sub-optimal dose (10 μg/ml) of MLBL. All the samples were analyzed by two-colour flow cytometry for their surface CD4 antigen expression versus intracellular IL2, IFNγ, IL4 and IL10 expression. Samples were analyzed by first gating on the CD4-positive population. The threshold for detection of positive cytokine-producing cells was set at 1% above the negative control consisting of cells treated with an irrelevant isotype-matched antibody. Results are expressed as means ± S.E.M. of 6 independent experiments. Each experiment used PBMC from a different subject.*

** P< 0.05*
***P<0.01 (Student's t test).*

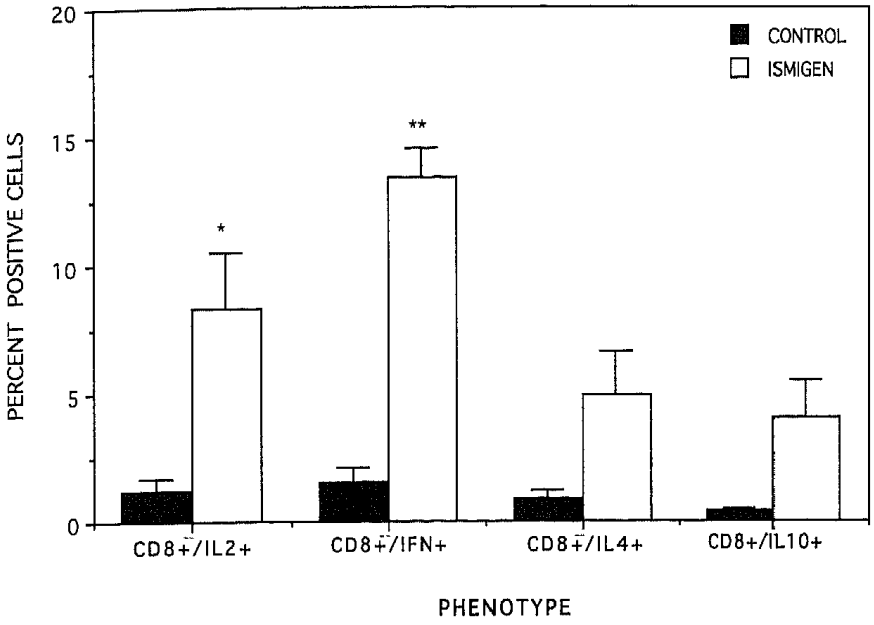

Fig. 6. *Effect of MLBL incubation on the frequency of CD8+ T lymphocytes producing IL2,IFNγ, IL4 and IL10 in re-stimulated cultures. PBMC were incubated for 120 hrs with 100 mg/ml MLBL and then re-stimulated with a sub-optimal dose (10 mg/ml) of MLBL. All the samples were analyzed by two-colour flow cytometry for their surface CD8 antigen expression versus intracellular IL2, IFNγ, IL4 and IL10 expression. Samples were analyzed by first gating on the CD8-positive population. The threshold for detection of positive cytokine-producing cells was set at 1% above the negative control consisting of cells treated with an irrelevant isotype-matched antibody. Results are expressed as means ± S.E.M. of 6 independent experiments. Each experiment used PBMC from a different subject.*

*$P<0.05$
**$P<0.01$ *(Student's t test).*

expansion and effector cell differentiation.

The results also showed that MLBL can both modulate innate immunity and induce primary immune response by direct activation of the production of different cytokine patterns by immune competent cells. In fact, the treatment of PBMC with MLBL increased both the frequency of CD4+ T cells producing IL2 and IL10 and the frequency of CD8+ T cells producing IL-2, IL-10 and IFNγ. Moreover, cytokine detection by ELISA in culture supernatants showed an increase in the concentrations of IL-2, IL-10, IL-12, and IFNγ, in respect to control cultures.

All these cytokines play a major role in the defence mechanisms of the organism in response to bacterial infections. In particular, in the supernatants of MLBL treated cells, the most represented cytokine is IL-12, a cytokine mainly produced by activated macrophages in response to many microbial products (30) that has a central role in the initiation of cell-mediated immunity and in the linking of the responses of the innate resistance and adaptive immunity (31). It is known, in fact, that IL-12 exerts a strong inducing effect on IFNγ production by NK cells (32), thus enhancing innate immunity responses and favouring the resistance to acute microbial infections. In addition, it is also an essential cytokine governing the differentiation of Type 1 responses mediated by T lymphocytes.

The other major Type 1 cytokine that we found both in culture supernatants and expressed in MLBL-activated CD8+ T cells was the IFNγ. This cytokine is known to exert two key functions: i) macrophage activation and subsequent enhancement of their antimicrobial activity; ii) stimulation of the production of IgG antibodies against Fcγ receptors and complement proteins that are the major antibodies involved in microbial opsonization and phagocytosis (33-34).

Finally, the data showed that the Type 2 cytokine IL-10 was largely produced by both CD4+ and CD8+ T cell subsets in MLBL-stimulated cultures. Also, this cytokine exerts an important antimicrobial activity, as it is known to activate B lymphocytes humoral responses and to be involved in the production of secretory IgA (sIgA) at the mucosal surfaces.

Taken together, the results on lymphocyte activation and cytokine production induced by treatment with MLBL show that, in addition to the activation of the innate immune responses through monocyte-induced release of IL-12, MLBL can also activate the specific cellular and humoral immune response pathways.

In a series of experiments, PBMC were incubated

with MLBL (100 μg/ml) for 5 days and then re-stimulated with a sub-optimal dose of MLBL (10 μg/ml) for 24 h. The results showed that MLBL induced the generation of antigen-specific effector T lymphocytes, as demonstrated by the increase, in both CD4+ and CD8+ T lymphocytes, of the rate of cytokine-producing cells after MLBL re-stimulation. In particular, in re-stimulated PBMC cultures, CD4+ T cell subset showed an increased percentage of cells expressing Type 2 cytokines (IL-4 and IL-10) in respect to both non-restimulated MLBL-treated cells and to MLBL-restimulated CD8+ T cells. This finding supports the hypothesis that MLBL re-stimulation could activate a secondary immune reaction recruiting a subset of effector CD4+ helper T cells secreting Type 2 (Th2) cytokines that may in turn activate B cell responses (17, 18, 20, 21, 35), thus indirectly providing an efficient help for immunoglobulin synthesis allowing a protective humoral immunity (11, 16, 22). As known, effector lymphocytes consist in antigen-experienced cells characterized by the capacity for production of very large amounts of multiple immunoregulatory cytokines upon re-stimulation with a low dose antigen (35-38). The enhanced secondary T cell response is due to the increased frequency of antigen-specific cells generated during the primary stimulation and to the cytokines present in the initial 5-days culture period that determine the developing cytokine profile of the effector populations (39). All these cellular immune functions, in concert with antibody responses, form the basis for protective immunity against infections and diseases (40-42).

The finding that MLBL re-stimulation generates effector lymphocytes that have a rapid cellular and humoral immune response, represents an important mechanism of action that may explain the potential of MLBL to elicit protective immunity also in recurrent infections of the respiratory tract thus supporting its role as a vaccine.

In their whole, the present results suggest that the therapeutic effect of MLBL in acute and recurrent infections of the respiratory tract is related to its capacity to elicit innate and adaptive immune responses, both in humoral and cellular compartments, and that this effect may be either by direct activation of immune competent cells or mediated through the generation of immune effectors. These results also support the results of previous clinical investigations that linked the therapeutic efficacy of the sub-lingual delivery of lyophilized bacterial lysates both in acute (22) and recurrent (12, 13, 22) infections of the respiratory tract to the stimulation of the immune response.

In conclusion, mucosal immunization by bacterial lysates appears as a non-invasive vaccine approach that does not require the use of extraneous adjuvant and that, beside cost-effectiveness, has attractive practical and immunological features.

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