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Independent Regulation of Antigen Processing and Presentation on Induction of Antibody Responses to Various Bacterial Antigens in C3H/He Mice

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Induction of antibody production in C3H/He mice by bacterial infection is regulated through the processing exerted by antigen presenting cells. From the studies with *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Micrococcus luteus*, lipopolysaccharides (LPS) in Gram negative bacteria, which are known to be T-cell independent B cell mitogen, seem to be the major factor stimulating immune responses via activation of macrophages. Activation of macrophage, however, does not seem to correlate with antibody production. *M. luteus* was easily eliminated by activated macrophages, while the processed antigens were immediately released into culture medium before presentation. Nevertheless, antigens from Gram positive bacteria, *Staphylococcus aureus* and *Bacillus subtilis*, were very active in chemotaxis and activation of peritoneal macrophages as well as in antigen presentation, while the very nature of the antigens is not yet clearly understood.

Key words: LPS, macrophage, chemotaxis, antigen presentation

Unlimited number of different antigens are detected on the surface of bacterial cells which in turn induce unique immune responses upon infection. Immune response is regulated both by host and by infected bacterial cells. A series of antigen processing and presentation is carried out by antigen presenting cells (APCs), where MHC polymorphism is largely responsible for the whole process. Correlationship between antigen presentation and increase in Ia molecules had been reported (22). Increase in Ia expression in macrophages and B cells is normally stimulated by IFN-γ, TNF-α, and IL-4, while down-regulated by PGE_2 and LPS (3, 4, 12, 13, 18), implying that immune response is regulated by various cytokines produced by immune cells upon bacterial infection.

Among various activated immune cells, macrophages are usually the first cells to eliminate bacterial cells upon their infection. The rate of phagocytosis seems to vary according to the antigen profile of the infected bacterial cells. Fast elimination by activated macrophages, however, does not necessarily stand for good antigen presentation.

Macrophages normally produce a variety of cytokines activating neighboring immune cells either antigen-specifically or non-specifically. Antimicrobial factors were identified against Gram positive (5) and Gram negative bacteria (21). Three kinds of proteins (Murine Macrophage Protein; MUMPs) from RAW264.7 were of bactericidal effects on *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, and others (8). Intracellular antibactericidal activities are always detected inside phagocytes as well. Immunomodulation of infected bacterial cells is also important in regulation of overall immune response in host.

Pathogenic bacteria, however, usually exert some disturbing activities on host immune system or they can avoid immune cell attack. Each pathogenic bacterium has a very unique mechanism of survival either by secreting immune suppressants or by modifying the surface antigenic structure. Leukocidin is produced by *Staphylococcus* sp. destroying the host immune cells (1), while streptococcal M protein can interfere the macrophage function and inhibit the production of cross-reactive antibody as well (11, 16). LPS from Gram negative bacteria also inhibit complement activation via alternative pathway (9). If *S. aureus* is capsulated, it is much less susceptible than non-capsulated to phagocytosis in the presence of normal

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rabbit serum, revealing that capsule is protecting peptidoglycan layer from antibodies (13) and inhibiting complement activation (9).

Immunologically active molecules on bacterial surface have also been reported. LPS, a bacterial endotoxin, stimulates IL-1 and IFN production from macrophages as well as the non-specific activation of murine B lymphocytes (2, 24). In contrast, MDP (N-methylmuramyl-L-ananyl-D-isoglutamine or muramyl dipeptide) is immunologically stimulatory (7, 19).

In this report, we demonstrate that the efficiency of bacterial antigen processing through phagocytosis is not necessarily correlated with the efficacy of antigen presentation and in subsequent antibody production.

Materials and Methods

Microorganisms and experimental animals

Three Gram negative bacteria and two Gram positive ones used in this experiment were *Micrococcus luteus* (ATCC 4698), *Pseudomonas aeruginosa* (ATCC 25619), *Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* and *Bacillus subtilis* (kindly provided by Prof. E.C. Choi, College of Pharmacy, Seoul National University). They were cultured in TSB (Tryptic Soy Broth, Difco) until the phase of exponential growth, washed in PBS for three times, and finally fixed with formaldehyde for an hour. After thorough wash, bacterial cells were freeze-dryed and powdered. Each aliquot of bacterial cell suspension at 1 mg/ml concentration was used throughout the experiment.

A mouse strain of C3H/He was originally obtained from the Core Breeding Unit of Prof. S.K. Paik, at Choongnam National University, and kept at Animal Resource Facility of Seoul National University thereafter. Mice with 20 g of body weight or slightly more were used for the experiment and provided with a nutritionally balanced rodent diet (Samyang Animal Feed, Seoul) and water *ad libitum*, and maintained under the standard condition.

Peritoneal macrophages

Peritoneal macrophages from previously injected with Brewer thioglycollate medium (Difco) were obtained in HBSS containing heparin at 10 unit/ml according to Weir and Herjenberg (29). They were washed several times in HBSS and kept in RPMI 1640 medium (Biological Industries, Israel) supplemented with 10% heat inactivated FBS (Gibco) and Gentamicin at the final concentration of 50 μg/ml (Flow Labortory). If necessary, adhesive cells were collected with cell scraper (Costar) after keeping them in tissue culture flask (Falcon 3024) at 37℃ for 2~3 hrs.

Splenocytes

Splenocytes were harvested as single cells through 60 mesh sieve. RBCs were eliminated in hypotonic solution (0.83% NH_4Cl and 10 mM HEPES), and splenocytes were washed twice in HBSS and cultured in RPMI 1640 as previously described (14).

Immunoglobulin secreting cells (ISCs)

Each group of three mice was injected i.p. with separate preparations of each bacterial cells. The control group was injected with PBS only. Mice were sacrificed at serial time points (days 3, 6, and 9). Immunoglobulin secreting cells were analysed by reversed hemolytic plaque assay as described by Gronowicz and Melchers (6). In short, 20 μl each of Protein A (Pharmacia Fine Chemicals) coated SRBCs prepared as 30% solution in HBSS, the same volumes of appropriately diluted rabbit anti-mouse IgGAM (Cappel) and Guinea pig complement (Gibco), and 100 μl of splenocytes at 4×10^5 cells/ml in HBSS were added to 0.3 ml of 0.4% agar solution (Bacto-agar in HBSS; Difco) supplemented with DEAE-dextran (Sigma) at the final concetration of 1 mg/ml, and poured onto plates. Plates were kept at 37℃ overnight in a 95% humidified CO_2 incubator (Forma). Plaques were counted and expressed as the number of immunoglobulin secreting cells per given numbers of the initial cells.

Splenic Index (S.I.)

Six groups of three mice each injected with bacterial preparation as described above were examined for splenic index on days 3, 6 and 9, with the control mice injected with HBSS only. Splenic index was calibrated as follows;

$$\text{S.I.} = \frac{\text{(weight of spleen in experimental group/ body weight of experimental group)}}{\text{mean of (weight of spleen in control group/ body weight of control group)}}$$

Measurement of specific antibody to bacteria

Specific antibody was titrated by ELISA on 96 multiwell flat-bottom titration plate coated with each of bacterial lysate prepared by ultrasonication (Labline System, Labline Instruments, Menrose Park, IL). Antisera obtained by heart-puncture were diluted to 1 : 100 in PBS supplemented with 1% BSA (Sigma). After 1 hr reaction and washing five times in PBS containing 0.5% (v/v) Tween 20, each well was developed with 10 ml of *o*-phenylene diamine (5 mg tablet, Sigma) in 0.1 M citrate buffer (pH 5.0) and 10 μl of 30% hydrogen peroxide.

Specific antibody reaction was read at 490 nm (Thermomax, Molecular Devices).

Phagocytosis of bacterial cells

Bacterial cells were labeled with FITC (1 mg/ml, Sigma) at 4℃ following Mock and Hancock (15) with slight modification. Unbound FITC was removed by repetitive centrifugation followed by washing with PBS supplemented with 1% BSA. The clearance of unbound FITC was confirmed by comparing fluorescence intensity of supernatant with that of washing media (PBS). Adhesive peritoneal macrophages were prepared as described. Total of 10^6 macrophages in 1 ml medium was mixed at 37℃ with FITC labeled bacterial cells in 6 well plates, and remaining cells in supernatant were removed from different wells at 30, 60, and 90 min of incubation, respectively. Macrophages were collected and washed five times and the relative intensity of phagocytosis was analysed with Flow Cytometer (FACStar^{PLUS}, Becton Dickinson).

Kinetics of phagocytosis

Each well of 96 multi-well plate was filled with 100 μl of peritoneal macrophages at 1×10^6 cells/ml of phenol red free RPMI 1640 medium. The plate was kept at 37℃ and turbidity of each well was monitored every half an hour with multi-well plate reader (Molecular Devices) at 590 nm. Stability of bacterial cells in macrophage culture supernatant was also evaluated in the same way. Bacterial cells were added at the final concentration of 1 mg/ml to 6 hr culture supernatant of macrophage, and its stability was continuously monitored incubating the plate at 37℃. Each group was assayed in triplicate. HEL (Hen egg lysozyme, Sigma) at 1 mg/ml was compared for lysing activity of bacterial cells with culture supernatant of macrophage. The activity was measured by the same method.

Six groups of three mice were injected with 100 μl of bacterial cells and peritoneal macrophages were taken out on day 3, 6, and 9, and *in vivo* phagocytosis of bacterial cells were analysed as described.

Detection of released antigens after processing

FITC labeled bacterial cells were added to 10^6 macrophages in 24 well plates, and incubated at 37℃ for 1½ hr. Plates were washed and released bacterial antigens preiviously labeled with fluorescence were detected, with further incubation, at 0, 1, and 2 hr at 37℃ with fluorospectrophotometer (UV-160, Shimadzu).

Quantification of LPS from bacterial cells

LPS on Gram negative bacterial cells were analyzed using anthrone test according to the method of Tamura *et al.* (20). In brief, 200 μl of bacterial cells, prepared as previously desribed, was mixed thoroughly with 1 ml of anthrone solution at 4℃, and boiled in water-bath for 10 min. Absorbency was measured at 625 nm with spectrophotometer. LPS from *E. coli* 055 : B5 (Sigma) was used for standardization.

Proliferation of immune cells

Total of 200 μl of splenocytes at 4×10^6/ml was added to each well of 96 multi-well plate and incubated for 1 hr at 37℃ in a humidified incubator before the addition of bacterial cells at the final concentration of 100 and 50 μg/ml each. After 48 hr incubation, MTT (3-(4,5-dimethyl thiozol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) was added to each well and further incubated for another 3~4 hrs. Supernatant was collected by centrifugation of the plate at 1,500 rpm for 5 min, and formazan was solubilized by the addition of 100 μl 0.04 N HCl-isopropanol. Absorbency was evaluated at 540 nm. LPS from *E. coli* 055 : B5 was used at 2.5 and 5 μg/ml each for positive control, and PBS for negative one.

Proliferation of B lymphocytes was detected by the same method. B lymphocytes were separated from adhesive cells by incubation in culture flasks for appropriate time and from T cells using the mixture of culture supernatant of J1j.10 (ATCC TIB 184) which is reactive with thy1.2 and complement obtained from rabbit sera. Viable cells after trypan blue counting were adjusted to 4×10^6/ml and 200 μl each was transferred to 96 multi-well plate where bacterial cells at final concentration of 100 and 50 μg/ml each were added in triplicate. For T lymphocyte proliferation, culture supernatant of J11d.2 (ATCC TIB 183) was used instead.

Evaluation of antigen presenting activities

Macrophages induced with Brewer thioglycollate medium were reacted for 6 hr with bacterial cells and fixed with 1% para-formaldehyde for 20 min after removal of remaining bacterial cells. Six groups of three mice each were injected with antigen-specifically fixed antigen presenting cells or with antigen presenting cells only. Splenocytes were obtained 7 days after sensitization and reactive T cells were enumerated by MTT proliferation assay. PBS injected group was used as a negative control while mice stimulated with 5 μg/ml of Con A as a positive control group.

Results

Immune Responses in mice injected with bacterial cells

With the injection of bacterial cells, mice showed spe-

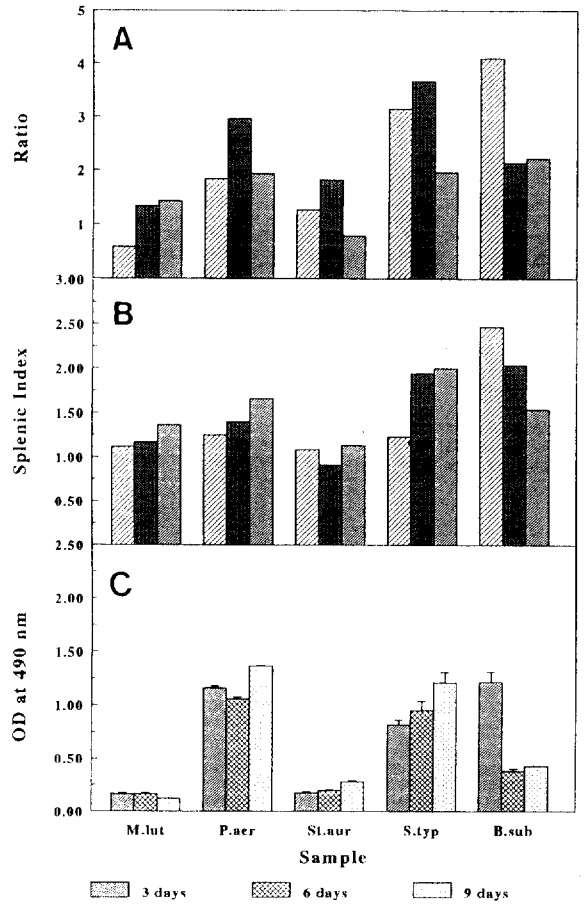

Fig. 1. Stimulation of immune responses by various bacterial antigens. Each group of mice was injected i.v. with bacterial cells on day 0 and sacrificed at serial time points (days 3, 6, and 9). A, frequency of antibody-secreting cells in spleen compared by the ratio of the induced plaque numbers to that of PBS injected group on the given days; B, splenic indices obtained from the same mice used in A; and C, the amount of antigen-specific antibody production detected in 1 : 100 diluted antisera raised with each of bacterial antigens (Mean± S.D.).

cific antibody production to the specific bacterial cells sensitized. The numbers of immunoglobulin secreting cells were counted on different days and immune response was expressed as the ratio of antibody production in antigen sensitized animals to immune responses on the same days of the control group injected with PBS only. As shown in Figure 1A, antibody production induced with the injection of bacterial antigens could be detected from day 3 in groups injected with *P. aeruginosa, S. typhimurium, St. aureus* and *B. subtilis*, while, with *M. luteus*, it could be shown only on day 6. In general, antibody production was sufficient enough only in groups with *P. aeruginosa, S. typhimurium*, and *B. subtilis*.

Immune response was also measured by splenic index (S.I.) as summarized in Figure 1B. The tendency of antibody production to specific antigens on given days is generally correlated with S.I. in all groups even if the intensity of response for each antigen may not be much proportional. The specific antibody production (Figure 1C) also shows the same trend with the high titers in three bacterial antigens as in Figure 1A.

In vivo phagocytosis of bacterial cells

When bacterial cells were introduced into peritoneal cavities, each of bacterial preparation had a unique kinetics of take-up pattern by phagocytosis. Bacaterial cells were labeled with FITC and they were recovered from phagocytes isolated from the peritoneal cavities at 30, 60, and 90 min after injection. Intensity of fluorescence from phagocytosed bacterial cells were measured and transformed into dry weight of total cells. *M. luteus* and *B. subtilis* were removed very quickly from peritoneal cavity, while the others such as *P. aeroginosa, St. aureus*, and *S. typhimurium* were not easily cleared (data not shown). It has been also confirmed when total fluorescence inside phagocytes was detected by flow cytometer at the same intervals as shown in Figure 2.

Phagocytosis of bacterial cells was detected *in vitro* at 37℃ in 96 multi-well plate reader. Equal amount of different fixed bacterial cells was added to the wells of phagocytes already prepared in a multi-well plate and clear-up was monitored in each well at 590 nm with 1 hr interval for 3 hr after initiation of the experiment and with 2 hr intervals, thereafter, until 21 hr (Figure 3). Decrease in optical density in *M. luteus* again was very steep and continued until 21 hr of the experiment, while the slopes for other bacteria were rather plain.

Detection of processed antigens

Bacterial cells are decomposed inside phagocytes, and processed antigens appear on the surface, and some of them are released spontaneously. Released antigens were detected using FITC labeled bacterial cells as above. Antigen release after process was relatively high in *B. subtilis*. The fluorescence intensity from other released bacterial antigens were not very high even with the incubation for 3 hr (Figure 4).

Infiltration of phagocytes induced by injected bacterial cells

Chemotactic effect of the bacterial cells inducing phagocytes, resulting in appropriate immune responses, thereafter, when introduced into the peritoneal cavity, was detected by calculating the total number of cells in the peritoneal cavity on day 3, 6, and 9 after injecting bacterial cells. Results were summarized in Figure 5. Chemotaxis was high in groups with *S. typhimurium* and *B. subtilis*. *P. aeruginosa* induced significant number of

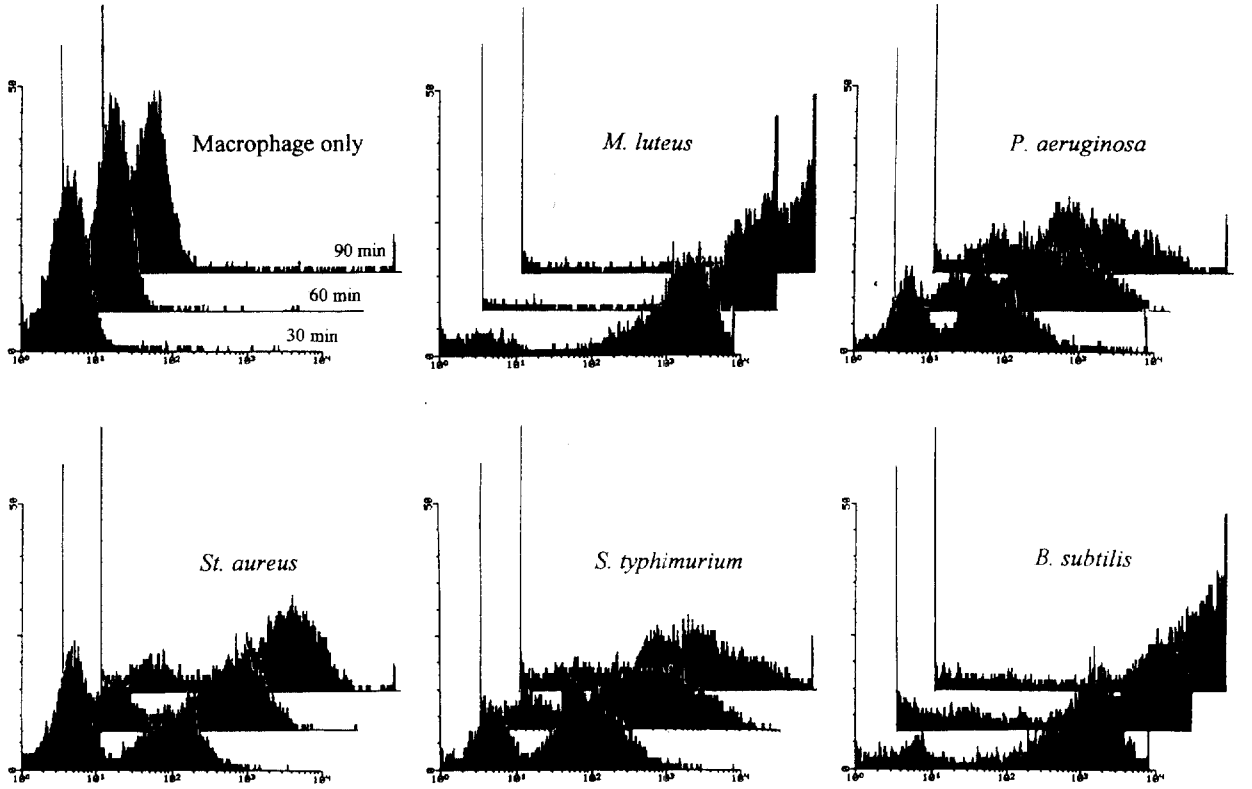

Fig 2. Analysis of active phagocytes with the stimulation of bacterial antigens. Each aliquot of various bacterial cell suspension was labeled overnight with FITC at 4℃ before phagocytosis. The numbers of active phagocytes and the intensity of activation were evaluated by the numbers of the cells among 5,000 cells examined on the basis of their fluorescence intensity.

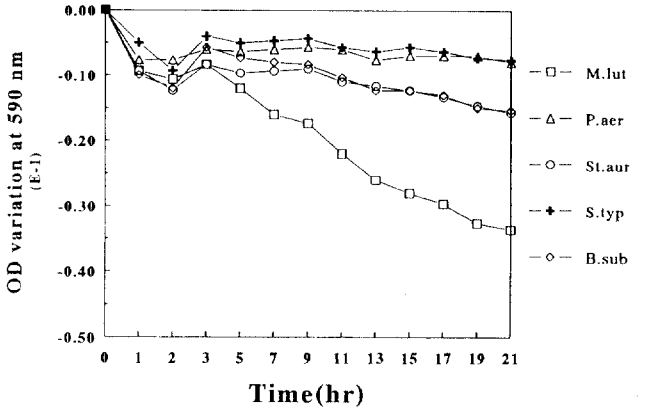

Fig. 3. Kinetics of bacterial take-up by macrophages. Bacterial cells at 100 μg in 100 μl of phenol-red free RPMI 1640 media in each well were added to 1×10^6 cells of macrophage in 96-well plate followed by incubation at 37℃. Turbidity of each well was read at 590 nm every hour (Background subtracted).

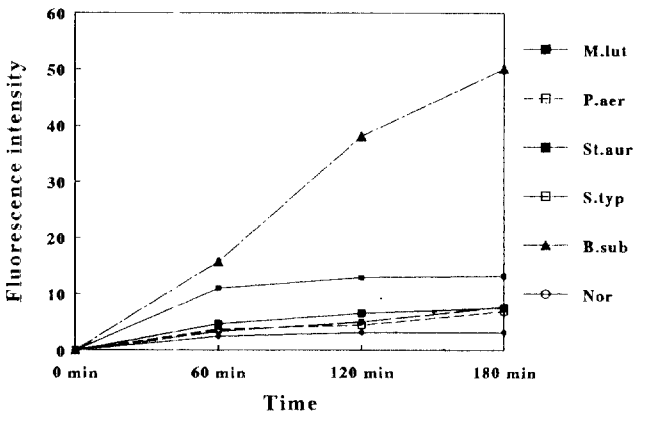

Fig. 4. Spontaneous release of antigens after antigen processing. Macrophages were allowed to take-up the labeled antigens for 90 min, and released antigens were detected by the fluorescence intensity from the culture supernatant of reaction mixture. The experiment was performed at 37℃ in triplicate. Data from the other experiments with antigen take up at 37℃ and release at 4℃ or with antigen take up at 4℃ and release at 37℃ are not shown. Nor stands for Normal

phagocytes into peritoneal cavity, while the induction with *M. luteus* and *St. aureus* was not statistically significant.

Proliferation of immune cells

Proliferative effect on unseparated spleen cells and separated B and T lymphocytes from spleens stimulated *in vitro* with different numbers of bacterial cells was evaluated by MTT method (Figure 6). The results show-

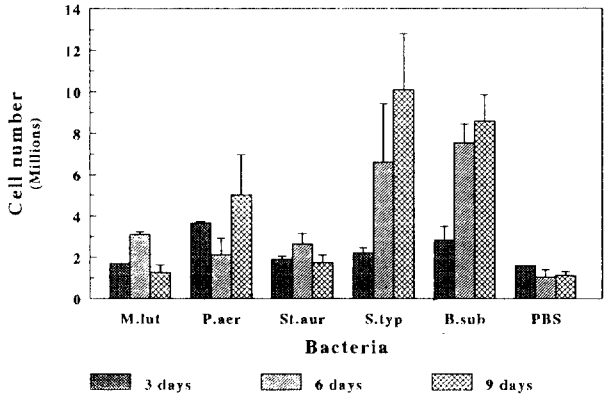

Fig. 5. Infiltration of macrophages into the peritoneal cavity induced by bacterial antigens. Chemotactic effect of bacterial antigens upon injection into the peritoneal cavity was examined by counting the total numbers of the macrophage infiltrated into the peritoneal cavity on given days.

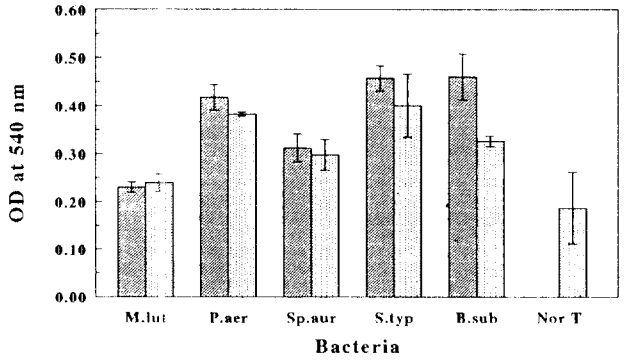

Fig. 7. Proliferation of primed T lymphocytes in the presence of antigen-presenting macrophages *in vitro*. Previously antigen-primed T lymphocytes were cultured either in the presence (▨) or in the absence (▤) of fixed antigens on antigen presenting cells prepared as described in Materials and Methods.

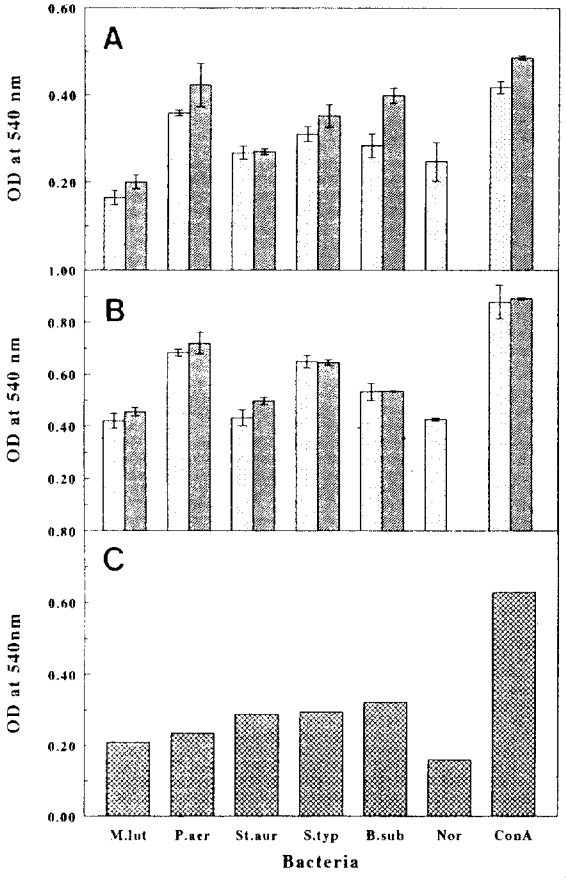

Fig. 6. Proliferation stimulated with bacterial cells *in vitro*. Proliferation of total spleen cells (A), separated B lymphocytes (B), as well as T lymphocytes (C) upon stimulation with bacterial cells. Total spleen cells and separated B lymphocytes were stimulated either with 50 μg (▤) or with 100 μg (▨), or separated T lymphocytes with 100 μg (▩) of bacterial cells, respectively.

ed that that Gram negative bacteria were relatively more stimulatory as shown in *in vitro* experiments. With separated B lymphocytes, very similar effects of bacterial stimulation were observed suggesting that B lymphocytes were stimulated by LPS from Gram negative bacterial cells. The stimulation of separated splenic T lymphocyte proliferation, however, was not significantly different among bacterial strains (Figure 6C).

Antigen presenting activities

Antigen presenting cells either with or without antigens were added to the culture of T lymphocytes from spleen previously immunized with bacterial cells, and the proliferation of T lymphocytes were regarded as the antigen specific immune response induced by bacterial antigen fixed on antigen presenting cells. Stimulatory effect of *B. subtilis* antigens on APCs, in comparison with APCs without bacterial antigens, was much more clear than those of other bacterial antigens, implying that the efficiency of processing and presentation of *B. subtilis* antigen was much higher and that the antibody production was strongly stimulated by APCs (Figure 7).

Discussions

Antibody production induced by *B. subtilis* was relatively higher than those by the other bacteria used in this experiment. In general, Gram negative bacteria was more stimulatory in induction of antibody production. LPS from Gram negative bacterial cell wall could have stimulated directly B lymphocytes or simply used as a T-independent antigen. Variation of the Gram positive bacteria in the stimulation of antibody production seems that they may have different stimulation mechanisms from the beginning of the immune response.

Induction of antibody production in Gram negative bacteria is mediated by LPS activating not only macropha-

ges and B lymphocytes but also LPS-reactive subsets of T lymphocytes (23). This may explain the high production of antibody by *B. subtilis* stimulation. Recognition, processing, and presentation of antigens are all important steps for specific antibody production. The result of ELISA showed that specific antibody production was high with the stimulation of *B. subtilis* (Figure 1C). This could be due to the efficient processing and presentation of antigens.

Recognition and take-up of bacterial antigens by activated macrophages was analyzed using flow cytometer. It seems that it does not take long to clear up bacteria by macrophages as shown in Figure 2. There is no increase of take-up of bacterial cells by macrophages after 30 min incubation. The tendancy of clear up may be dependent on the different nature of various antigens. It seems that there must be a kind of clear cut for the activation of phagocytes according to the nature of antigens, showing the numbers of active phagocytes is much unlikely to increase throughout the experiments. As shown in Figure 3, even if the phagocytic take-up of bacterial antigens occurs very rapidly, there are still many bacterial cells in the supernatant, and the whole take-up progress seems to take over relatively slowly instead of the intensity in phogocytes measured by flow-cytometer. Phagocytosis, however, does not necessarily explain whole immune response, since it is known as a non-specific immune response.

Bacterial antigens are released from the phagocytes after processing. They may be reabsorbed by other phagocytes, but immunogenicity of those antigens is low as shown in *Listeria monocytogens* (22). Fluorescence labeled bacterial antigens were detected after phagocytosis and analysed of their antigenicity. Secretion of bacterial antigen was high in phagocytes with bacterial cells which they could easily take-up. They did not have high antigenicity as expected when analysed by dotblotting with the mouse immune-sera. Nevertheless, released antigen such as N-formyl peptide derived from the surface protein could induce chemotaxis for neutrophils regulating early immune responses by modifying the population of immune cells in peritoneal cavity. Flow cytometer analysis of peritoneal cells after stimulation with *B. subtilis* revealed the increase of polymorphonuclear cells up to 10%, while there was no significant increase with the other antigens.

Bacterial cells could be broken by the extracellular enzymes secreted by phagocytes. Antigen stability on the surface of phagocytes was measured using the variation of optical density. The antigens from *M. luteus* was relatively unstable while most of the antigens from other bacteria remained rather intact until the end of the experiment (data not shown). It was not clearly identified what phagocytes released into the culture supernatant, but it seems that this could be another possible mechanisms of removing bacterial antigens in the body.

LPS content in Gram negative bacteria may be important in inducing the infiltration of macrophages into the peritoneal cavity in dose dependent way (17). The total amounts of LPS in *Ps. aeruginosa* and *S. typhimurium* are different inducing the proportional macrophage infiltration (Figure 5).

The stimulation of B lymphocyte proliferation was directly affected by the bacterial antigens while proliferation of T lymphocyte was increased slightly only regardless of the antigenic nature. This means that T lymphocytes may be further activated via APCs after processing.

Presentation of *B. subtilis* antigen by APCs was very effective, when compared with those of other antigens, inducing high production of antibody. High efficiency of phagocytosis and presentation could have stimulated T lymphocyte activation inducing the production of cytokines required for the antibody production by B lymphocytes. Different immune responses could be induced by the different nature of antigens.

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