PROLIFERATION RESPONSES IN PREIMMUNIZED MICE LYMPHOCYTES BY BORDETELLA PERTUSSIS CELL WALL COMPONENTS

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ABSTRACT

Bordetella pertussis infects the respiratory tract of the human host and causes whooping cough in children. The nature of immunity against Bordetella pertussis infection and disease is poorly understood. The aim of this study was to investigate cell mediated immunity in mice immunized with outer membrane component of cell wall, of B. Pertussis.

A group of mice were immunized with outer membrane complex (OMC) and killed whole cell (WCV) of B. pertussis, with an interval of 2 weeks. During a period of 7 weeks following the immunization, lymphocytes were isolated from lymph nodes of immunized mice. The in vitro proliferative response of isolated lymphocyte to stimulation with 20 μg of 30 and 69 kDa outer membrane protein (OMP) were measured as parameters for cell mediated immunity (CMI). The data were expressed as mean count per minute (CPM)×10³ after subtraction of the CPM of unstimulated control cultures. Lymphoblastogenic response was observed in immunized mice with WCV and OMC. At 30 days of post immunization a significant increase in response to 30 and 69 kDa OMP was observed, a small decrease in the response was evident against P30 and P69 at 60 and 120 days of post immunization, but the response was still higher than what was observed in control mice.

Current findings indicate strongly the potential of outer membrane protein component of B. pertussis in proliferating lymphocytes in the mice.

Keywords: Bordetella pertussis, Cell mediated Immunity, OMC, WCV, Proliferation assay.
Proliferation Response of Cell Wall Component of B. Pertussis

INTRODUCTION

* Bordetella pertussis* is the main etiological agent of whooping cough, a severe respiratory disease that mainly affects infants and young children, although adults can also become infected (1). Currently available vaccine consisting of killed whole cell *pertussis* organisms can cause local and systemic side effects (2,3). Concern about the reactogenicity of whole-cell vaccines (4) led to the development of acellular *pertussis* vaccines, which are composed of defined purified antigens, designed to achieve protection while minimizing adverse side effects (5).

The nature of immunity against *B. pertussis* is poorly understood. Research on protection has traditionally been focused on the role of humoral pertussis specific antibodies (6), because *B. pertussis* had been considered to be an extracellular pathogen, but no clear correlation has been found between serum antibody levels and protection (7,8).

Several reports have shown that *B. pertussis* can survive in mammalian cells including macrophages (9,10). Animal experiments are providing increasing evidence that CMI may also be necessary for complete, long-term protection against *B. pertussis* (11,12). It has been suggested that T helper1 subset may be responsible for protection against respiratory *B. pertussis* infection in mice (12). Evidence from murine models suggests that control of *B. pertussis* respiratory infection can be better achieved by immunization schedules favoring type 1 cytokines production (13,14,15). Therefore, the mechanism that prevents replication and dissemination and eventually succeeds in eliminating *B. pertussis* from the body appear to reflect the dual extra and intracellular location of the bacteria in the host and require the distinct but coordinated function of the cellular and humoral arms of the immune response for optimal protection (15,16). OMPs being T-cell dependent immunogen, may in part contribute to the protection through cellular immune response as well. Bordetella *pertussis* 30 and 69 kDa OMP have been associated with virulent phenotype of *B. pertussis*. OMP is needed to be presented to the immune system by way of an outer membrane complex structure (17).

We performed the present study to investigate *pertussis* specific cell mediated immune responses in mice immunized with outer membrane complex (OMC) and to compare it with mice immunized with killed whole cell bacteria (WCV). As parameters for CMI, T lymphocyte proliferative responses in vitro were chosen. Lymphocytes from immunized mice were isolated from lymph nodes. The proliferative responses to stimulation with 30 and 69 kDa OMP were studied from 30 to 120 days after immunization.

MATERIALS AND METHODS

Female Balb/c mice (20 mice per group), 4-8 weeks, were used in all experiments. Freeze-dried *B. pertussis* bacteria (vaccine strain, Tohama 1) were diluted in saline and plated on Bordet-Gengou plates and incubated at 35°C for 72 hr.

Preparation of outer membrane complex: *B. pertussis* was grown in Verwey broth, the pellet was washed once with saline and resuspended into Tris-HCL pH 8.5. The suspension was sonicated on ice, after centrifugation at 10,000 g, the supernatant was pelleted for 1h at 100,000g. The pellet was resuspended in 1% sarcosine and centrifuged for 100,000g (17).

Preparation of whole killed bacteria: *B. pertussis* was inactivated for 30 min at 56°C and centrifuged at 3000g for 1 hr.

Isolation of P30: Outer membrane protein fraction was treated with zwittergent (chaps) buffer and incubated in 37°C for 1.5 hr. The treated fraction was applied to DEAD sepharose CL-6B (Pharmacia) and Sephacryl S-200 (Pharmacia) column. The results of SDS-PAGE showed that the fraction containing P30 were considerably pure (17).

Isolation of P69: The 69 kDa protein was extracted by incubating the bacterial cells at 60°C for 1hr and precipitated by poly ethylene glycol (PEG) and acetone and purified in gel filtration column in advance (18).

Lymphocyte proliferation test: The mice were immunized with 5μg of OMC and 12×10^5 of WCV. After 30, 60, 120 days of immunization, lymph nodes were removed from immunized and non immunized mice, minced with a scalpel blade and gently passed through a Steel wire mesh, lymphocyte were prepared in RPMI 1640 medium...
(Gibco) supplemented with 100 IU of penicillin per ml, 50μg of streptomycin and 1mM glutamine (complete medium).

The cells were washed twice in complete medium and cultured in flat bottom microtiter plates (Nunc, Roskild) containing 2x10^5 cell per well in a volume of 200μl of complete medium in the presence of predetermined optimal dose of P30 and P69. All tests were carried out in triplicate. Culture was incubated for 4 days at 37°C in an atmosphere of 5% CO2 in humidified air. Twenty-two hours prior to harvesting, 1 μCi of [3H] thymidine (Amersham) was added to each well. The cells were harvested by fiberglass paper (Pharmacia) and the incorporated radioactivity was measured with scintillation counter. The data were expressed as mean counts per minute (CPM)x10^3 of triplicate cultures. Lymphocyte proliferation was defined as positive when the mean CPM was >3x10^3 after subtraction of the CPM of the unstimulated control cultures (19,14).

Statistical methods: Comparison of responses between group of mice was done with the t-student test. Two-way analysis of variance and LSD, using SPSS statistical software, compared the levels of proliferative responses at different times following the immunization with different antigens. Probability values below 0.05 were considered statistically significant.

RESULTS

Blastogenic response in mice immunized with whole killed cell of B. pertussis:

The lymphoblastogenic responses were observed in preimmunized mice with WCV against P30 and P69 and PHA. The lymphoblastogenic responses to P69 at 30 days after immunization with WCV was (SI: 8.12±0.48) and P30 (SI: 7.48±0.69) shown in fig 1 and table 1. The degree of response was much higher with P30 and P69 at 30, 60, 120 days after immunization, however at 30 days post immunization a significant increase (P=0.001, F: 37.53) in response to both P30 and P69 was observed. Conversely, a decrease in blastogenic response apparent to PHA at this time remained almost the same at 60, 120 days. A slight decrease in the level of response was evident against P30 and P69 at 60 and 120 days post immunization, despite the fact that the response was still higher than that observed at day zero.

Blastogenic response in mice immunized with outer membrane complex:

Lymphoblastogenic response in preimmunized mice with OMC against P30 and P69 and PHA was observed. The degree of response was much higher at 30, 60 and 120 days after immunization, however at 30 days post immunization a significant increase (P=0.001, F: 22.61) in response was observed. A slight decrease in the level of response was evident against P30 and P69 at 60 and 120 days post immunization (fig 2 and table 2).

DISCUSSION

Despite increasing evidence that T-cell mediated immune responses are important for complete termination of B. pertussis infections, however, there are no studies evaluating the significance of pertussis specific cell mediated immunity in relation to pertussis OMPs component.

CMI has been reported, both in murine models of B. pertussis respiratory infection and in children recovering from pertussis (13,19). Recent reports suggest that B. pertussis can be taken up and survived within alveolar macrophages. It is conceivable that Th1 type responses are needed to activate phagocytes to facilitate clearance of intracellular pathogens (20). Some studies indicate a Th1 response after natural infection and vaccination with whole cell vaccine, while vaccine consisting of PT (pertussis toxin) and FHA (filamentous heamagglutinin) tended to induce a Th2 type response (13).

To investigate the induction of a pertussis specific CMI after immunization with WCV and OMC, antigen specific T-cell response were measured. In a recent study pertussis specific CMI responses were assayed by in vitro proliferation assay of mice lymphocyte cells. The results of present study show that from 30 days after immunization of mice with WCV and OMC a strong T-cell- mediated reactivity to cell wall component of B. pertussis is developed. The significant proliferative responses from 30 to 120 days following immunization with WCV and
Proliferation Response of Cell Wall Component of B. Pertussis

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Before Immunization</th>
<th>30 days after Immunization</th>
<th>60 Days After Immunization</th>
<th>120 Days After Immunization</th>
</tr>
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<tbody>
<tr>
<td>P30</td>
<td>2.11±0.31</td>
<td>7.48±0.69</td>
<td>7.12±0.69</td>
<td>6.12±0.36</td>
</tr>
<tr>
<td>P69</td>
<td>1.78±0.81</td>
<td>8.12±0.48</td>
<td>7.98±0.31</td>
<td>7.1±0.78</td>
</tr>
<tr>
<td>PHA</td>
<td>4.98±0.27</td>
<td>4.1±0.49</td>
<td>4.38±0.78</td>
<td>3.99±0.49</td>
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</table>

Proliferative response of lymph node lymphocytes from immunized mice with \(12\times10^8\) WCV, in response to P69 and P30 OMPs of B. pertussis after 30, 60 and 120 days of immunization. The concentration of antigens were based on previous titration and were as follows; PHA (10\(\mu\)g), P69 (20\(\mu\)g), P30 (20\(\mu\)g). Lymphoproliferation values are reported as mean \(\text{SE±CPM}\times10^3\) of \(^{3}\text{H}\) thymidine incorporation, after subtraction of CPM of antigens unstimulated cultures.

Fig. 1. Proliferative response of lymph nodes lymphocytes from immunized mice with \(12\times10^7\) WCV, in response to P69 and P30 OMPs of B. pertussis after 30, 60 and 120 days of immunization. The concentrations of antigen were based on previous titration and were as follows; PHA (10\(\mu\)g), P69 (20\(\mu\)g), P30 (20\(\mu\)g). Lymphoproliferation values are reported as mean \(\text{SE±CPM}\times10^3\) of \(^{3}\text{H}\) thymidine incorporation, after subtraction of CPM of antigens unstimulated cultures. Thin bars show standard deviations.

OMC were found against 30 and 69 kDa OMP antigens. P30 and P69 are OMP antigens in B. pertussis. The response of P69 was higher than 30 kDa OMP antigen. The proliferative response were also found to be higher in immunized mice than in the non immunized. The specific proliferative responses to P69 and P30 proved that the lymphocytes react to these antigens. The proliferative response was maximal 30 days after immunization, a decrease in response was apparent to PHA at this time, remaining almost the same at 60, 120 days. A slight decrease in the level of response was evident against P30 and P69 at 60 and 120 days post immunization, despite the fact that the response was still higher than that observed at day 0.

Hamster found a correlation between mouse protection assay potency and the presence of OMP in outer membrane complex (17). OMC structure allows the OMP to remain in the native conformation and may supply adjuvant activity because of the presence of LPS (17). Ausiello
Table 2. Proliferative response of mice immunized with one

<table>
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<tr>
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</tr>
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<tbody>
<tr>
<td>P30</td>
<td>1.12±0.52</td>
<td>6.85±0.71</td>
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<tr>
<td>P69</td>
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<td>7.79±0.78</td>
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<tr>
<td>PHA</td>
<td>4.1±0.51</td>
<td>4.34±0.36</td>
<td>4.16±0.24</td>
<td>3.72±0.52</td>
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</table>

Proliferative response of lymph nodes lymphocytes from immunized mice with 5 μg OMC, in response to P69 and P30 OMPs of *B. pertussis* after 30, 60 and 120 days of immunization. The concentration of antigens were based on previous titrations and were as follows; PHA (10μg), P69 (20μg), P30 (20μg) Lymphoproliferation values are reported as mean SE±CPM×10^3 of [3H] thymidine incorporation, after subtraction of CPM of antigens unstimulated cultures.

![Graph showing proliferation response](image)

Fig. 2. Proliferative response of lymph nodes lymphocytes from immunized mice with 5μg OMC, in response to P69 and P30 OMPs of *B. pertussis* after 30, 60 and 120 days of immunization. The concentration of antigens were based on previous titration and were as follows; PHA (10μg), P69 (20μg), P30 (20μg) Lymphoproliferation values are reported as mean SE±CPM×10^3 of [3H] thymidine incorporation, after subtraction of CPM of antigens unstimulated cultures. Thin bars show standard deviation.

demonstrated a proliferation response to PT, FHA and P69 in healthy vaccinated adults (21). High proliferation responses have been reported against FHA and native PT toxin in immunized children (22). CMI against 69 kDa OMP has been demonstrated following immunization with whole cell pertussis vaccine in human (23) and mice (24).

Specific proliferation responses for native and recombinant P69 is supported by several studies in immunized human (21,22) and mice (11,15).

These preliminary data document for the first time that immunization with OMC induces a strong proliferation response to 30 and 69 kDa OMP antigens: 30 and 69 kDa OMP are active components of WCV and OMC as well (25).

The role of P30 in CMI against *B. pertussis* is still controversial, therefore in the present study we evaluated the proliferative responses of immunized mice to P30.

P30 like P69 was potent mitogen for murine cells as well. Our study showed that proliferative response against these antigens persisted until 120 days after immunization. These data suggest that Outer membrane complex of *B. pertussis* may have an important role in inducing cell mediated immunity. We have shown that OMP antigens might be involved in CMI against *B. pertussis* following immunization of mice with OMC. So the combination of PT and FHA with the active component of OMPs antigens would be best candidate for inclusion in new pertussis vaccine in future. Further studies will be needed for the assessment of cytokines profile to purified OMP antigens of *B. pertussis*.

ACKNOWLEDGMENTS

This work was supported by a grant from Tarbiat Modarres University.
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REFERENCES


