Proliferate Resonse to Purified *Bordetella pertussis* Toxin on Murine Spleen Lymphocytes

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Summary

A purification procedure of pertussis toxin (PT) from submerged culture of *Bordetella pertussis* (*B.pertussis*) strain 134 using adsorption and affinity chromatography was discussed. The yield of the resulting PT was approximately 37.5mg/l of concentrated culture supernatant. The polypeptide pattern of the purified PT was investigated by SDS-PAGE and showed five bands between 11 to 26 KDa using low molecular weight marker. Since PT is the main component of acellular pertussis vaccine, obtaining a good yield of it would be essential for production of the new and safer vaccine generation. The other objective of this study was to determine the effects of PT on murine lymphocytes using MTT test. The effect of various doses of prepared PT on murine lymphocytes showed that the amount of 0.5µg/0.1ml had the highest proliferation. Furthermore comparison between the resulting PT and phytohemagglutinin showed much higher effect of PT on murine spleen cells. These results indicate that *B.pertussis* strain 134 is a suitable strain for induction of PT in order to use it for development of acellular vaccine in Iran and also for *in vitro* studies on proliferation of murine lymphocytes.

Key words: pertussis toxin, purification, lymphocyte proliferation, spleen cell

Introduction

Pertussis toxin (PT) produced by *Bordetella pertussis* (*B.pertussis*), causative agent of whooping cough is a major toxin involved in the pathogenesis of this highly

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contagious respiratory disease and it is generally believed to play an important role in the induction of protective immunity (Edward *et al* 1999) during vaccination against pertussis. The toxin produces a whole range of biological activities when nanogram doses are injected into laboratory animals (Robinson *et al* 1985). As a result of these diverse effects, many names have been applied to PT such as histamine-sensitizing factor (HFS), islet activating protein (IAP) and lymphocytosis-promoting factor (LPF). The name LPF specifies a markedly enhanced leukocytosis due predominantly to increased numbers of circulating small lymphocytes (Robinson *et al* 1985). Furthermore, PT proved to be a potent mitogen for murine spleen or lymph node cells *in vitro* (Morse *et al* 1977, Petersen *et al* 1993). To study its biological activitis and for development of an effective and safer vaccine a simple method for purifing PT in large quantities has been need. Over two decades considerable progress has been made on the purification of PT including cesium chloride density gradient centrifugation, adsorption to hydroxylapatite, multi-step and single-step affinity chromatigraphy (Chong & Klein 1988, Robinson *et al* 1985, Perera 1970).

In this study we were purified PT from submerged culture of *B.pertussis* in high yield using affinity chromatography on fetuin-Sepharose 4B and measured proliferation effects of resulting PT on murine spleen lymphocytes and phytohemagglutinine in order to develop an experimental accellular vaccine using a local strain.

Materials and Methods

Bacterial strain and culture. The *B.pertussis* strain 134 used in this study was the routine pertussis whole cell vaccine production strain in Razi Institute (Karaj, Iran). The bacteria were grown in a modified Stainer-Scholte medium in a 45L fermenter at 35˚C for 40h. At the end of culture periode the purity of the culture was checked. Approximately 4.6×10^{10}CFU/ml of the bacterial cells was removed by

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centrifugation at 10000*g* for 30min at 4°C. Residual bacteria were taken out of the supernate by filtration through a 0.22µm filter (Seitz, Germany). The pH of supernatant was adjusted to 7.5 with 5N HCl and concentrated 20-fold by ultrafiltration (Sartocon 2000, Sartarius) then stored at -20°C.

Toxin purification procedure. PT was purified using one-step affinity chromatography according to the methode of Irons & MacLennan (1979). In this regard, a 5×15cm column of hydroxylapatite (Sigma) equilibrated in 0.01M phosphate buffer, pH8 at a flow rate of 500ml/h at 5°C was prepared. Then the concentrated culture supernatant was passed through it. Under these conditions, all the filamentous hemagglutinin (FHA), another *B.pertussis* surface antigen, bind to the hydroxylapatite while PT was passed through (Sato *et al* 1983). For binding PT the eluate was percipitated with ammonium sulphate added to 74% saturation. After several times centrifugations, the supernate was passed through fetuin-Sepharose 4B column. The column was prepared by dissolving fetuin (Sigma) in 75ml of coupling buffer (0.01M $NaHCO_3$, pH8.0, containing 0.5M NaCl) and added to 30ml of CNBr-activated sepharose 4B. After mixing the contents for 18h at 4°C, the gel was washed sequentially with 200ml of each of the coupling buffer, sodium acetate buffer, buffer C (0.1M Tris, pH10.0, containing 0.5M NaCl and 3M KSCN) and finally coupling buffer. The supernate was applied to a 2.5×10cm of fetuin-Sepharose, equilibrated in the phosphate-NaCl, pH7.2 buffer at a flow rate of 1ml/min at room temperature. Column was washed with 200ml of buffer A consisting of 0.1M Tris-HCl, pH7.5, including 0.5M NaCl and 200ml of buffer B containing 0.1M Tris-HCl, pH7.5, containing 2M NaCl at 4°C and flow rate of approximately 40ml/h. After washing the column with a further 200ml of buffer A, bound PT is eluted with 60ml of buffer C with a flow rate of approximately 10ml/h. The amount of 5ml of each fraction containing protein were pooled, neutralized with 1N HCl and dialyzed for 16h against 100 volume of PBS containing 0.02% thimerosal. The retentate containing PT was stored at -20°C. The total protein of the

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PT was estimated by Bradford method (Bradford *et al* 1976). The purity of the final PT was assessed by SDS-PAGE gel.

SDS-PAGE. This was performed according to Wardlaw *et al* (1976). Sample was prepared by mixing with an equal volume of solubilizing buffer and heating at 100°C for 5min. Separating and stacking gel contained 11% (w/v) and 5% (w/v) acrylamide respectively, both gels and buffer contained 0.1% (w/v) SDS. The gels were stained with a coomassie blue fixin-staining solution.

Assays of purified PT. The *in vitro* and *in vivo* techniques were employed for estimation of the toxin. Hemagglutinating activity of PT was assayed using chicken erythrocytes. Mitogenic effect of PT was determined using mice spleen cells. For the lymphocyte proliferation assay 8-12-week-old female C57 BL/6 mice were obtained from Razi Institute. The mice were killed by cervical dislocation and their spleens were suspended in RPMI 1640 (Sigma) supplemented with 5% heat-dislocation and isolated inactivated fetal calf serum (FCS), penicillin (100U/ml) and streptomycin (100mg/ml). The resulting cell suspensions were filtered with 200-number sieve mesh and washed three times with the medium and then centrifuged at 1000rpm/min for 10min repeatedly. The individual cell suspensions were dissolved in 2ml of complete medium solution; Trypan blue counting and cell concentration was regulated to 2×10^5/ml counted the number of viable cells. 100µl of cells suspension (approximately containing 20×10^3cells) were put into three rows of 96 well culture plate, the following agents were added put into each of three rows: various concentrations of purified PT (first row), phytohemagglutinin (PHA-L, Sigma) second row and the negative control with no agent (third row). The plate was incubated at 37°C and 5% CO_2 for 72h. The proliferative responses were determined on the basis of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion to Formazan as described by Mosman *et al* (1986). Four hours before the end of incubation time 10µl MTT solution (5mg/ml, Sigma) was added to each well and incubation for further 3-4h. The plate inverted rapidly to remove medium and

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100µl of organic solvent (Isopropanol/HCl) was added to each well of the plate. The plate was located on a shaker, agitated for 10-20min in order to solubilize Formazan crystals and was read at 570nm and 630nm as reference on spectrophotometer. The optical densities obtained were directly proportional to the percentage of living cells present in the each well. Proliferation index was defined as percentage of living cells divided into one hundred.

Results

High yield of PT in the culture supernatant of *B.pertussis* strain 134 was obtained. Culture of the strain in Stainer-Scholte medium under the explained conditions was resulted in approximately 37.5mg/l of the concentrated culture supernatant. The total protein estimation of PT was 1.2mg/ml.

SDS-PAGE. SDS-PAGE pattern of the purified PT from concentrated culture supernatant on the fetuin-Sepharose 4B column is shown in figure 1. Five bands corresponding to five PT subunits S1, S2, S3, S4 and S5 with 26, 22, 21, 12 and 11 KDa molecular weight respectively are detected.

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Figure 1. *SDS-PAGE profile of affinity-purified PT and its subunits. Lane 1:standard molecular weight, lane 2:30µg of fetuin sepharose affinity purified PT, lane 3:concentrated culture supernatant*

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Assay procedure. The amount of purified PT in the culture supernatant was determined by hemagglutinating activity with chicken erythrocytes.The recorded HA titer after one hour at room temperature, HA=6 (log_2), indicatedc that good yeilds of PT can be obtained from srain 134 under the experiment conditions. In another assay, various quantities of the purified PT were added to mouse spleen cells culture on row of 96 well microplate. Simultaneously PHA with the same concentrations was added to another row of cell cultures and the obtained results were compared (Table 1).

Proliferation index	Concentrations of purified PT and PHA (µg/0.1ml)									
	0.1	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**	**1.0**
PT	0.44	1.02	1.38	1.62	1.90	1.80	1.65	1.43	1.12	0.80
PHA	0.34	0.80	1.00	1.10	1.20	1.22	1.26	1.28	1.30	1.34

There was relatively a sharp increase in proliferation rate at 0.5µg dose of PT over 0.1ml of the spleen cells. The optimum concentrations were observed between 0.25 to 0.5µg after 72h, while the optimum dose for PHA was between 0.5-1.0µg (Figure. 2). Furthermore the effect of PT higher than the optimum dose showed suppression effect on murine lymphocytes proliferation and there was a cytotxic effect over 1.0 µg. The comparative ability of optimum amounts of the resulting PT and PHA to stimulate (20×10^3) C57 BL/6 spleen cells was tested for 72h of culture. Under these conditions it was found that the purified PT was much more potent than PHA as it is shown in figure 2.

Discussion

In this study we have applied a single-step purificatipon procedure using fetuin-Sepharose 4B affinity chromatography to produce PT from *B.Pertussis* strain 134 culture medium in good yeilds and high purity. The strain is a potent mitogen and capabel of inducing a mitogenic effect on mice spleen cells. The purification

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procedure allows preparation of purified PT in amount of approximately 37.5mg/l of concentrated culture supernatant. The appropriate selected strain and the purification method are two main factors that contribute to the success in PT production in high yeilds. In current study strain 134 was selected for production of PT on the basis of its capacity to produce toxin in the pertussis vaccine production procedure. The srain shows retarded growth in Stainer-Scholte medium and under the conditions employed good yeilds of PT can be obtained from strain 134.

Figure 2. *MTT test on the spleen cells. Various concentrations of the resulting PT and PHA were used and proliferation index of each was measured. A:the resulting PT, B:PHA*

On the basis of SDS-gel electrophoresis it can be indicated that pure PT (Figure 1, lane 2) is obtained by the applied method. The estimate of molecular weight of the protein found by the SDS-PAGE was similar to those reported by Sato *et al* (1988). Use of fetuin as an affinity matrix in one-step chromatography has some advantages in purification procedure in which this resin could be given homogenous toxin in high purity and yeilds, and reduced the operational cost of the large-scale production of PT. Another advantage of the purification procedure is that purified PT is essentially free of undesirable endotoxin (Chong & Klein 1989). And can be directly detoxified and used for vaccine production. Pertussis vaccine has a non-specific mitogenic effect on T-lymphocytes that is due to PT (Morse *et al* 1976, Anderson *et al* 1977). They found that at a concentration of 1-4μg/ml, PT was a

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potent mitogen for spleen or lymph node cells *in vitro* but not for thymus or bone marrow cells. PT exhibited a bimodal response, as at concentrations of less than 0.4µg/ml or more than 5µg/ml, it had no significant mitogenic action. The mitogenic action of PT for T cells at concentration of 3µg/ml after 72h has been demonstrated (Pizza *et al* 2003). Our results on murine lymphocytes showed PT optimal mitogenic action at concentration of 2.5–5µg/ml. This range was relatively equal to the range of Morse and his co-workers.

PT has a potential role in the development of new and safer pertussis vaccine such acellular vaccine has been prepared and evaluated (Ruuskansen *et al* 1991) in the volunteers. Here, we have purified PT using single-step fetuin-Sapharose 4B affinity chromatography to develop a suitable vaccine which capable of eliciting protective immune responses after passing standard quality and safety tests.

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