Effect of 65 kDa Buffalo Placental Protein on B-Cell Proliferation and Antibody Response

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ABSTRACT : Immunosuppressive potential of 65 kDa buffalo placental protein (bPP65) on B-cell proliferation *in vitro* and antibody response *in vivo* was evaluated. B-cell proliferation was estimated by measuring incorporation of tritiated thymidine in buffalo lymphocytes while primary antibody responses against phytohaemagglutinin (PHA) or keyhole limpet haemocyanin (KLH) were evaluated in mice. bPP65 suppressed proliferation of lipopolysaccharide (a B-cell specific mitogen)-stimulated buffalo lymphocytes *in vitro* indicating suppression of B-cells. This suppression was dose dependent over the protein concentration range 25-100 µg/ml. Primary antibody responses in mice against PHA and KLH in presence of bPP65 were lower as compared to in its absence but these were not statistically significant. Amino acid composition data of bPP65 and BSA suggested that bPP65 is different from BSA. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 3 : 432-437)***

Key Words : Placental Protein, Immunosuppressive Protein, Antibody Response, B-Cell Proliferation

INTRODUCTION

The placenta of mammals is an active endocrine organ secreting numerous proteins, prostaglandins and steroids. Some of these placental products have been implicated as having immunomodulatory action at fetomaternal interface to contribute to non-rejection of the fetus inheriting genes from father as well as mother. Immunosuppressive proteins, such as placental protein-14 (PP14), placental protein-15 (PP15), pregnancy specific β-1 glycoprotein (SP1) and pregnancy zone protein (PZP) have been identified from human placenta (Bohn et al., 1982; Bohn et al., 1980; Hau, 1986; Tatarinov and Masyukevich, 1970). PP14 from human placenta is well-characterised immunosuppressive protein, which mediates immunosuppression through lower production of IL-2 and perhaps IL-1 as well (Pockley and Bolton, 1990; Pockley and Bolton, 1989). Althouh presence of immunosuppressive proteins has been established in primates (Germain et al., 1989; Tease et al., 1989), placental trophoblast of bovine (Hansen, 1997; Newton et al., 1988) and ovine (Newton et al., 1989), uterine milk secretions of bovine (Segerson and Bazer, 1989) and ovine (Hanson et al., 1989; Hanson et al., 1987; Segerson et al., 1984), culture supernatants of embryos of horse (Roth et al., 1990; Watson, 1990), rabbit (Pandian et al., 1988), ovine and porcine (Murray et al., 1987), mechanism of immunosuppression is not worked out. Our earlier studies (Mehta and Rajput, 1999) have shown that buffalo placenta exhibits immunosuppressive activity when tested *in vitro* using phytohaemagglutinin (PHA) induced lymphocyte proliferation. Further, it has been established that a buffalo placental protein with a molecular weight of 65 kDa is associated with immunosuppressive effect (Mehta and Rajput, 2001). Although, non-rejection of fetus is largely mediated through T-cells, contribution of suppressed primary antibody response can not be ignored. The primary antibody response requires involvement of both B and T-cells and in the present study, the effect of 65 kDa buffalo placental protein (bPP65) on B-cell proliferation *in vitro* and primary antibody response *in vivo* has been investigated. Since, the molecular weight of this protein is close to the molecular weight of bovine serum albumin (BSA), amino acid composition of these two proteins were compared in order to ascertain the identity of bPP65.

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MATERIALS AND METHODS

Materials

RPMI-1640, phenyl methyl sulphonyl fluoride (PMSF), lipopolysaccharide (LPS), phytohaemagglutinin (PHA), keyhole limpet haemocyanin (KLH), ficoll-400, sodium diatriazoate, bovine serum albumin (BSA), carbonic anhydrase, fetal calf serum and standard amino acid mix were procured from M/s Sigma Chemical Co., USA. Tritiated thymidine (^{3}H-TdR, specific activity 50 mCi/mmol) was purchased from Board of Radiation and Isotope Technology, Mumbai. Sodium pyrophosphate, 2, 5-diphenyloxazole (PPO) and toluene were from Loba Chemical Co. Mumbai. PVDF membrane (Westran) was purchased from Sehleicher and Schuell Membrane Filters, USA.

Placenta was collected from Murrah buffalo maintained at Institute's Cattle yard.

Preparation of placental homogenate

Placental homogenate was prepared as described earlier (Mehta and Rajput, 1999). Pacenta just after parturition was

brought to the laboratory. Cotyledons were separated from rest of the placenta and were cut into small pieces, and homogenized in ice-cold phosphate buffered saline (PBS) containing 0.1 mM PMSF using pestle motor and acid washed sand. The homogenate was centrifuged at 10,000×g for 20 min. at 5°C and the supernatant so obtained was extensively dialyzed (3 changes, 100 vol. overnight) against PBS containing 0.1 mM PMSF at 5°C. The dialyzed samples were stored at -20°C till further use.

Purification of 65 kDa buffalo placental protein (bPP65)

bPP65 was purified using preparative SDS-PAGE, electroelution and ultrafiltration techniques (Deepthi, 2000; Mehta and Rajput, 2001). Proteins from placental extract were separated in 3 mm thick 10% acrylamide gel in SDS-PAGE. While gel adhering to glass plate, two gel strips (one each from each ends) were cut vertically. The protein bands in these strips were stained with $CuCl_2$ (Harlow and Lane, 1988). Stained strips were aligned to adhered-gel on the glass plate. Horizontal gel-strip from unstained gel corresponding to 65 kDa protein was cut and protein was eluted using electroelution assembly. SDS present in electroelution buffer was removed in ultrafiltration system using 10 kDa cut-off membrane. Homogeneity of bPP65 was checked using SDS-PAGE (Laemmli, 1970) and native PAGE.

Protein estimation

The protein content of the samples was estimated by using dye-binding method of Bradford (1976) using BSA as a standard.

Lymphocyte preparation

Lymphocytes were isolated using Ficoll-paque (Boyum, 1968). Ficoll-paque solution was prepared by dissolving 5.7 g Ficoll-400 and 9 g sodium diatrizoate per 100 ml of distilled water for obtaining a density of 1.077 g/ml of resultant solution. Blood collected into heparinized tubes from the jugular vein of the buffalo calf was mixed with equal volume of RPMI-1640 medium. Diluted blood was layered onto Ficoll-paque in the round bottomed sterile disposable tubes (12×75 mm) and centrifuged (2,500×g, 20 min, 25°C). The buffy layer containing lymphocytes at the interface was removed and diluted three times with RPMI-1640 medium. The lymphocytes were pelleted (2500×g, 20 min, 25°C). The pellet was treated with erythrocyte-lysis buffer (17 mM Tris-HCl, pH 7.2 containing 144 mM NH_4Cl) for 60 sec. and centrifuged (2,500×g, 20 min, 25°C). The lymphocytes were washed once in RPMI-1640 and finally resuspended in RPMI-1640 containing 20 µg/ml LPS (Pockley and Bolton, 1990) and 15% FCS, at a concentration of 1×10^6 cells/ml.

Measurement of B-cell suppression

B-cell proliferation was evaluated by measuring incorporation of tritiated thymidine (^{3}H-TdR) in LPS-induced (Pockley and Bolton,1990) buffalo lymphocytes. 0.1 ml lymphocyte (1×10^6 cells) cells were distributed to each well of 24 well culture plate (Costar, USA). bPP65 samples were aseptically added at the initiation of the culture. In control cultures, instead of bPP65 samples, equal volume of PBS was added. All treatments were carried out in quadruplicate. The cells were cultured for 96 hr at 37°C in a humidified atmosphere of 5% CO_2. Subsequently 0.1 µCi (25 µl) ^{3}H-TdR was added to each well of 24-well culture plate. After 24 h of ^{3}H-TdR addition, the mononuclear cells were detached from the surface of the plate and were centrifuged at 3,000×g for 5 min. The supernatant was discarded and cell pellet was suspended in 1 ml 5% TCA containing 30 mM Sodium pyrophosphate. The resultant solution was passed through glass fiber filters (GF/3, 25-mm dia.) and washed with atleast 20 ml methanol and 20 ml distilled water employing water suction. The filters were placed in scintillation vial and air dried (overnight). Seven ml of scintillation fluid (6 g PPO/lit of toluene) was added to each vial and counts were taken for one minute using β-counter. Incorporation of ^{3}H-TdR into responding cells was determined by averaging the dpm values from quadruplicate wells. Results were expressed as percentage suppression of ^{3}H-TdR incorporation relative to the control.

Measurement of primary antibody response

The effect of bPP65 on primary antibody response was studied in two different sets of experiments using PHA or KLH as immunogens using the method described by Veselsky et al. (1996). For each set of experiment sixteen male albino mice (3 months old) were randomly selected. In set I eight mice in experimental group were given an intraperitoneal injection of 0.2 mg bPP65 on days 0 and 1. Eight mice in control group, instead of bPP65, saline was administered. On day 2, in both experimental and control groups, the mice were immunized with 250 µg of KLH. In set II experiment was conducted similar to set I except KLH was replaced with PHA. On day 10, mice were sacrificed and blood was collected from heart. Antibody titres against immunogens (KLH or PHA) were measured by ELISA (Barman and Rajput, 1994; Smith and Wilson, 1986) which is briefly described here. Each well of 96-well ELISA plate (Costar, USA) was coated with 1 µg KLH or PHA dissolved in 100 µl of PBS. Coating was done at 5°C overnight. Each well was then washed with PBS-Tween 20 (0.05%). Blocking was achieved by filling the wells with BSA (1%)-PBS-Tween 20 (0.05%) solution at room temperature for 2

h with occasional shaking. After removing blocking solutioin, 100 µl antisera (different dilutions) were added and incubated for 2 h at room temperature. Each well was then washed four times with PBS-Tween 20 (0.05%). After washing, 100 µl of anti-mouse immunoglobulin-peroxidase conjugate diluted 1:500 with BSA-PBS-Tween -20 were added and plates were kept for 2 h at room temperature. Each well was again three times washed with PBS-Tween 20 and two times with PBS. After final washing, 100 µl substrate (4 mg o-phenylenediamine dissolved in 10 ml of 50 mM sodium citrate, pH 5.0 containing 0.01% H_2O_2) was added and plates were incubated for 30 min. Reaction was stopped by 100 µl 4N H_2SO_4 and absorbance was recorded at 490 nm in ELISA plate reader.

Amino acid analysis

Protein samples were hydrolyzed after their binding to PVDF membrane. Ten pieces (2×2 mm each) of membrane were activated by dipping in 100% ethanol for 15 sec, then washed in water for 15 min and equilibrated with buffer (2.5 mM Tris, 19.2 mM glycine, pH 8.2) for 5 min. The activated and washed membrane pieces were immersed in 1 µg/µl protein solution (prepared in equilibration buffer) for 2 min., washed several times with distilled water and placed at the bottom of borosilicate glass tubes (5 × 100 mm). Then, 300 µl of 6N HCl containing 0.1% phenol (redistilled) was added. Nitrogen was bubbled above the solution for 5 min. and sealed. Sealed tubes were kept at 110°C for 24 h (Hugli, 1989). The hydrolyzed samples were centrifuged (10,000×g, 2 min). Sample drying, derivatization with phenyl isothiocyanate (PITC) and separation of amino acid derivatives were carried out as per the instructions of manufacturers using Water's HPLC system. For control sample, PVDF membrane devoid of protein sample, was treated in similar way and values, if any, obtained for individual amino acids were subtracted from experimental values. Carbonic anhydrase was used as standard for checking the reliability of the method.

In brief, 250 µl of protein hydrolysate was placed into capillary tubes and vacuum dried. Then, 25 µl of redry solution (methanol: water: triethylamine in 2:2:1 ratio) was added, vortexed and vacuum dried. This step was repeated once again. Then, 20 µl of derivatising agent (methanol: water: triethylamine: PITC in 7:1:1:1 ratio) was added, contents vortexed and left for 5 min. at room temperature and vacuum dried. To these capillary tubes, 100 µl of diluent (prepared by mixing 95 ml of 4 mM $Na_2HPO_4{\cdot}2H_2O$, pH 7.4 and 5 ml of acetonitrile) was added and vortexed. The reverse phase column Bondapak C-18 was equilibrated with 90% eluent A (prepared by mixing 940 ml of 1.5 M sodium acetate trihydrate containing 0.05% triethylamine, pH 6.4 and 60 ml of acetonitrile) and 10% eluent B (60% acetonitrile). 20 µl of sample was injected into column and following gradient system was used for separation of amino acid derivatives.

Time (min)	Flow (ml/min)	%A	%B
Intial	1.00	100	0
10.00	1.00	54	46
10.50	1.00	0	100
11.50	1.00	0	100
12.00	1.50	0	100

Statistical analysis

The data was expressed as the mean±SE. Student's t-test was used and p value <0.05 was considered significant.

RESULTS AND DISCUSSION

The SDS-PAGE profiles of proteins extracted from buffalo placenta and bPP65 purified by using preparative SDS-PAGE, electroelution and ultrafiltration are shown in figures 1a and 1b. It is clear from figure that bPP65 is electrophoretically homogenous confirming our earlier observations (Mehta and Rajput, 2001).

The effect of different concentrations (25 µg/ml to 100 µg/ml) of bPP65 on LPS-induced-buffalo-lymphocyte

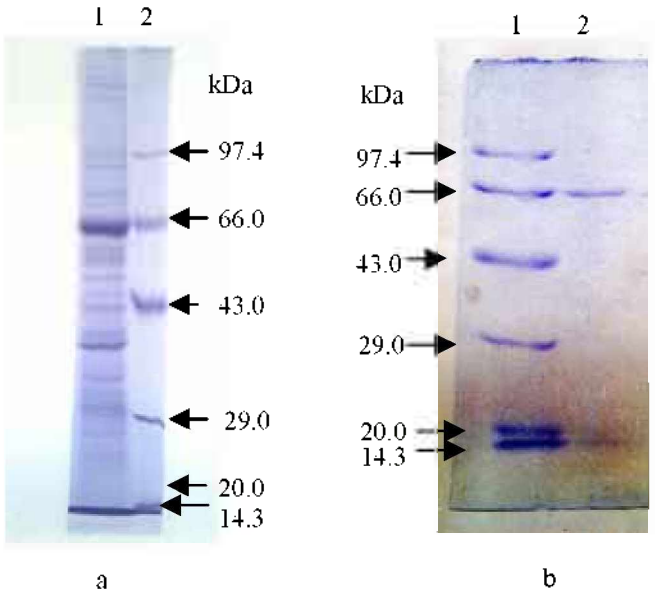

Figure 1. SDS-PAGE profile of buffalo placental proteins (Figure 1a) and bPP65 (Figure 1b). Proteins were separated in 10% acrylamide gel and stained with coomassie brilliant blue. Figure 1(a). Lane 1, Placental proteins (50 µg); Lane 2, Molecular weight markers (15 µg). Fig.1(b). Lane 1, Molecular weight markers (25 µg); Lane 2, bPP65 (4 µg).

Table 1. Dose response of bPP65 on LPS-stimulated buffalo lymphocytes

Amount of bPP65[#] (μg/ml)	dpm* (Mean± S.E.M.)	Percent inhibition
0	728.3±55.3	0
25	615.4±39.5	16[ab]
50	503.7±19.7	31[ac]
75	438.3±12.6	40[ad]
100	344.4±31.1	53[ad]

* Mean of quadruplicate cultures from one experiment.
[#] LPS at a concentration of 20 μg/ml.
[a] Unpaired t-test.
[b] Non-significant.
[c] Significant, $p<0.05$.
[d] Significant, $p<0.01$.

proliferation *in vitro* is shown in table 1. It is clear that suppression by bPP65 is dose dependent. At 75 μg/ml and 100 μg/ml bPP65 concentrations, the suppressions in proliferation of LPS-induced-lymphocytes were 40% and 53% respectively and this suppression was significant ($p<0.01$). However, at 50 μg/ml bPP65 concentration, the suppression was significant at 5% level. The present results demonstrate that bPP65 suppresses LPS-induced-buffalo-lymphocyte proliferation *in vitro*. Since LPS (mitogen) specifically proliferates B-lymphocytes, the suppressive effect of bPP65 is mediated through B-lymphocytes. No contamination of other cells in lymphocyte preparation was observed (figure 2). There are no reports where suppression of LPS-induced lymphocyte proliferation by placental proteins has been evaluated.

Figure 2. Lymphocytes in culture in RPMI-1640 medium containing 15% FCS and LPS (20 μg/ml)

Our earlier *in vitro* studies have shown that immunosuppressive effect of bPP65 is mediated through T-lymphocytes (Mehta and Rajput, 2001). A similar mechanism is believed to be operating for placental proteins (PP14, PP15, PZP) from human. In all these studies, T-cell specific mitogen, PHA, has been employed. Further it is generally believed that cell mediated anti-fetal immunity is the one which is compromised while antibody response is usually dominant. B-cell proliferation is inhibited in presence of bPP65 indicating involvement of B-cell in lowering immunological response of pregnant mother towards fetus.

The influence of bPP65 on primary antibody response in mice against two different antigens viz. PHA and KLH is reported in table 2 and table 3 respectively. Antibody levels at different dilutions of antiserum were compared in order to know the effect of bPP65 on primary antibody response. Although it appears from the table that at serum dilutions equal or higher than 1:3000, the antibody production against both the antigens was lower in presence of bPP65, but these differences were statistically non-significant.

Although bPP65 *in vitro* suppresses the B cell (buffalo) proliferation, but it did not show significant lowered antibody production in mice against both PHA and KLH antigens. The possibility that bPP65 acts in a species specific way can not be ruled out. On the other hand, immunosuppressive protein from boar seminal plasma has

Table 2. Effect of bPP65 on antibody response against phytohaemagglutinin (PHA)

Antisera dilution	Anti-PHA antibody level* (PHA is used as immunogen in absence of bPP65, Mean±S.E.M.)	Anti-PHA antibody level* (PHA is used as immunogen in presence of bPP65, Mean±S.E.M.)
1:100	0.28±0.00	0.27±0.02 (0)[ab]
1:500	0.20±0.02	0.20±0.03 (0)[ab]
1:1.000	0.16±0.02	0.14±0.02 (10)[ab]
1:2.000	0.12±0.02	0.10±0.02 (13)[ab]
1:3.000	0.13±0.03	0.08±0.02 (39)[ab]
1:4.000	0.09±0.02	0.06±0.01 (26)[ab]
1:5.000	0.08±0.02	0.06±0.01 (27)[ab]
1:6.000	0.07±0.02	0.05±0.01 (28)[ab]
1:7.000	0.06±0.01	0.04±0.01 (36)[ab]
1:8.000	0.05±0.01	0.03±0.01 (41)[ab]

Figures in parenthesis indicate percent suppression of anti-PHA antibodies by bPP65.
* Antibody signal as measured by absorbance at 490 nm.
[a] Unpaired t-test.
[b] Non-significant.

Table 3. Effect of bPP65 on antibody response against keyhole limpet haemocyanin (KLH)

Antisera dilution	Anti-KLH antibody level* (KLH is used as immunogen in absence of bPP65. (Mean±S.E.M.)	Anti-KLH antibody level* (KLH is used as immunogen in presence of bPP65. (Mean±S.E.M.)
1:500	0.48±0.04	0.49±0.01 (0)[ab]
1:1,000	0.44±0.04	0.44±0.03 (0)[ab]
1:2,000	0.41±0.04	0.38±0.04 (7)[ab]
1:3,000	0.37±0.05	0.34±0.04 (8)[ab]
1:4,000	0.35±0.05	0.31±0.03 (12)[ab]
1:5,000	0.30±0.05	0.25±0.03 (17)[ab]
1:8,000	0.30±0.05	0.22±0.03 (27)[ab]
1:10,000	0.28±0.05	0.20±0.03 (29)[ab]
1:12,000	0.22±0.05	0.17±0.03 (23)[ab]
1:14,000	0.23±0.05	0.16±0.02 (31)[ab]

Figures in parenthesis indicate percent suppression of anti-KLH anbtibody by bPP65.

* Antibody signal as measured by absorbance at 490 nm.

[a] Unpaired t-test.

[b] Non-significant.

been shown to suppress primary antibody response in mice (Veselsky et al., 1996). Of course, this protein is from different species and also not from placenta. As immunological processes are interdependent, it is difficult to quantify the contribution of lowered cell mediated and humoral response by bPP65 for survival of fetus.

The molecular weight of bPP65 is approximately 65 kDa (Mehta and Rajput, 2001) which is identical to molecular weight of BSA. Thus, in order to differentiate bPP65 from BSA, amino acid composition data of bPP65 and BSA were compared. Amino acid composition of these two proteins was determined and results are shown in table 4. Amino acid composition for most of the amino acids of these two proteins is different from each other suggesting that these two proteins are different. The amino acid composition of carbonic anhydrase (used as standard protein) was in close agreement with the reported amino acid composition values for this protein (table 4) indicating reliability of the method used for determination of amino acid composition. Our earlier studies (Mehta and Rajput, 2001) have shown that isoelectric point of bPP65 is 6.4 and is a glycoprotein. This also confirms that bPP65 is different from BSA.

Table 4. Amino acid composition of bPP65

Sr. no.	Amino Acid	Number of residues				
		bPP65	BSA*		Carbonic anhydrase[#]	
			Theoretical[a]	Observed[b]	Theoretical[a]	Observed[b]
1.	Met	13	4	2.5	3	2.4 (1)
2.	Cys	2	-	-	-	-
3.	Val	53	36	37.1	20	18.2 (19)
4.	Tyr	9	19	21.1	8	6.7 (7)
5.	Pro	55	28	25.5	19	17.7 (11)
6.	Arg	28	23	27.6	9	7.7 (7)
7.	Thr	27	34	29.1	14	10.0 (11)
8.	Ala	39	46	46.0	17	17.0 (17)
9.	His	29	17	13.5	11	6.2 (11)
10.	Glu	94	79	60.4	23	19.8 (29)
11.	Ser	28	28	18.7	16	10.4 (9)
12.	Gly	52	16	17.8	20	20.8 (24)
13.	Asp	19	54	28.3	32	22.3 (27)
14.	Lys	20	59	30.0	18	13.5 (17)
15.	Leu	33	61	62.1	26	24.2 (21)
16.	Phe	-	27	27.9	11	9.1 (9)
17.	Ile	15	59	30.0	5	4.6 (5)

* Data for BSA are included for comparative purpose.

[#] Data for carbonic anhydrase for checking reliability of the method followed. Figures in parenthesis indicate amino acid composition data from present investigation.

[a] Theoretical values.

[b] Observed values using acid hydrolysis and PITC derivatisation.

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