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A protein in pig spleen similar to immune suppressive protein of stress

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KEY WORDS immune function; lymphocytes; stress; spleen

ABSTRACT

AIM: To purify a protein in pig spleens, which was similar to immune suppressive protein of stress (ISPS), and characterize its properties and functions. **METHODS:** 1) Pig spleen was extracted in dilute hydrochloric acid. 2) The extract was ultra-filtrated for having high molecular weight proteins (M_r>30 000). 3) The filtrates were purified with FPLC affinity chromatography. 4) The elute from FPLC was used for T-lymphocyte proliferation and ELISA test. 5) Lastly, SDS-PAGE was used to determine the molecular weight and purity of the final product. **RESULTS:** A protein purified from pig spleen (the pig ISPS homologue) inhibited concanavalin A (Con A)-induced mouse lymphocyte proliferation. The molecular weight of this protein was about M_r 190 000. It has a stronger selectivity against T-lymphocyte line such as Jurkat cell line and mastocyte line (P815) and has a weaker inhibitory activity on macrophage line (U937). **CONCLUSION:** A protein similar to rat/mouse ISPS was found in pig spleen. This may provide an opportunity to study its roles in tumors and autoimmune diseases.

INTRODUCTION

In our previous work, we reported that the immune suppressive protein of stress (ISPS) from restraint stressed rat/mouse serum could suppress normal lymphocyte proliferation[1-3]. We also reported that this protein inhibited certain immune functions such as the release of IL-1 from T-lymphocyte and delayed supersensitivity. Further experiments showed that ISPS was produced in peripheral lymph tissue (lymph node, spleen, and intestinal tract). By HPLC, the molecular weight of ISPS was approximately 190 000[4-6]. The results from SDS-PAGE suggested that ISPS may be consisted of two or three subunits and studies are currently underway to explore its amino acid sequence. A monoclonal antibody against ISPS (2C4) was successfully generated and used for affinity chromatography to purify ISPS from our group.

One of the biggest technical restrictions of our previous work was to harvest large amount of ISPS from small animals like rats and mice. This limited our ability to further characterize ISPS. In the present study we have found that pigs are better resources of ISPS. This will not only reduce the cost and technical demand of sacrificing large number of mice and rats, but also simplify the purification process. We further characterized the properties and functions of this pig ISPS homologue.

MATERIALS AND METHODS

Culture medium RPMI-1640 (BioConcept,

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Umkirch, Germany) supplemented with *L*-glutamine 2 mmol/L, 10 % calf serum (BioConcept, Umkirch, Germany), benzylpenicillin 100 kU/L, and streptomycin 100 mg/L (HuaBei Pharmacy stock corporation, China) were used.

Materials 2C4 hybridization Neoplasm cells[7] were provided by the Neuroimmunne Laboratory of Beijing University. Horse-radish peroxides coupled sheep anti rat IgG and Biotin coupled sheep anti-mouse IgG were purchased from Sigma Chemical Company (St Louis, Missouri, USA). AKTA FPLC chromatography apparatus was provided by Pharmacia (Amersham Pharmacia Biotech AB company, Australian). Fresh pig spleens were obtained from the Beijing Abattoir.

Pig ISPS homologue extraction Fresh pig spleens were processed by first being homogenized in 0.05 mol/L (1:10, 1 g fresh tissue/10 mL HCl) and centrifuged at 15 000×*g,* 4 °C for 20 min. The supernatant was lyophilized and resuspended in phosphate buffered saline (PBS 0.01 mol/L, pH 7.4). The suspension was run through the Ultrafree-500 Filter Device Gradocal (Millipore Corporation, USA). Proteins with high molecular weight (M_r>30 000) were retained, collected, and resuspended to the original volume with PBS (0.05 mol/L, pH 8.0).

Preparation of 2C4-McAb After removal from liquid nitrogen the cells of 2C4-McAb cell line were quickly defrosted at 38 °C for 1-2 min and mixed with 5-8 mL RPMI-1640 medium. The solution was centrifuged at 200×*g* for 10 min. The precipitate was suspended in RPMI-1640 medium and incubated at 37 °C in 5 % humidified CO_2/95 % atmosphere.

The BALB/c mice were immuno-enhanced by injecting 0.5 mL pristine intraperitoneally. Ten days after pristine injection, each mouse was ip injected with 0.5 mL RPMI-1640 containing 1×10^6 tumor cells. The ascites were collected one week later. The collection was centrifuged (200×*g*) at 4 °C for 10 min and the supernatant was collected. Equal volume of saturated $(NH_4)_2SO_4$ solution (pH 7.0) was mixed with the supernatant and the mixture was stored overnight.

The mixture was centrifuged (15 000×*g*) at 4 °C for 20 min the next morning. The precipitant was resuspended with PBS (0.01 mol/L, pH 7.4) to achieve 1/5 of the original volume. The sample was then dialyzed against PBS (0.01 mol/L, pH 7.4) until there was no apparent $BaSO_4$ sediment in the dialysate. The dialysis-purified sample was adjusted to pH 8.0 (PBS, 0.01 mol/L) and then (2 mL) purified through the Hitrap protein A column (17-0717-01 Pharmacia). The column was washed with PBS (0.01 mol/L, pH 8.0) to remove non-column binding protein. After a multi-protein washout peak detected by UV absorbance curve at 280 nm, acetate buffer (0.1 mol/L, pH 3.0) was used to elute the 2C4-McAb monoclonal antibody.

2C4-McAb coupled FPLC affinity chromatography column Above 2C4-McAb was concentrated by ultra filtration (a membrane with molecular weight cut off of M_r 30 000); the protein concentration was determined by the method of Bradford[8], and the final concentration was adjusted to 15 g/L. To couple 2C4-McAb to the protein A beads, the fresh protein A column was firstly washed to remove isopropanol in the column with 6 mL of cold HCl (l mmol/L) at a flow-rate of 1 mL/min, and 1 mL of 2C4-McAb (15 mg) was injected into the column and stored at 4 °C for 4 h, followed by washing with buffer B (6 mL, acetate 0.1 mol/L including NaCl 0.5 mol/L, pH 4.0). Buffer A (6 mL, ethanol amine 0.5 mol/L, NaCl 0.5 mol/L, pH 8.3) was then added and kept in the column for 15-30 min. The column was treated again with buffer B and A twice followed by washing the column with 2 mL neutral buffer PBS (0.01 mol/L pH 8.0) for balancing the pH. Finally the column was washed with 4 mL NaH_2PO_4 solution 0.05 mol/L (including 0.1 % sodium azide) and stored in 20 % alcohol.

Pig ISPS homologue purification by FPLC The 2C4-McAb monoclonal antibody coupled column was washed with PBS buffer (0.05 mol/L, pH 8.0) at a flow of 0.5 mL/min and monitored with UV 280 nm detector. After the UV absorption curve is stabilized, 2 mLof sample was injected into the column, incubated for about 1 h, and then washed with PBS buffer (0.05 mol/L, pH 8.0). Multi-protein peak (the protein without binding to the column) appeared. When the UV absorption curve returned to basal line, citric acid (0.1 mol/L, pH 3.0) was used to elute the pig ISPS homologue-2C4-McAb complex from the column. The UV absorption peak fraction was collected, the protein concentration was measured and the protein activity was tested.

Lymphocyte proliferation The spleen lymphocytes from normal BALB/c mice were collected and suspended in RPMI-1640 mediums. The cells were cultured in 96-well flat-bottom plates at 2×10^9 cells/L. Concanavalin A (Con A, 2 mg/L or 4 mg/L) and various concentrations of pig ISPS homologue and multi-protein washout were added to the lymphocyte cultures. Each experimental condition was repeated in 3 wells

(triplicate) and the result was averaged. The cultures were incubated at 37 °C in a humidified atmosphere of 5 % CO_2. After 42 h of incubation, [^{3}H]thymidine (TdR, 7.4 kBq/well) was added to each well, and the cells were then harvested onto glass filter paper after an additional 6-h incubation. Radioactivity assay was performed in a liquid scintillation counter and the radioactivity was recorded in Bq units.

ELISA The spleen extract was separated with ultra-filtration (a membrane with molecular weight cut off of 30 000) into two fractions. Both of fractions (M_r>30 000 and <30 000) were tested by ELISA. In brief, 96-well plates were coated with the extract (100 µL/well, 5 mg protein/L) in carbonate buffer (50 mmol/L, pH 9.5). The primary antibody (2C4-McAb, 100 µL, 0.3 mg/L) and the secondary antibody (sheep anti-mouse Ig, 100 µL/well, 1:1000) were added. Before optical densitometry, OPD (*O*-phenylenediamine, 0.04 mg/well) was added for secondary antibody staining. The *A* value was measured under optical densitometer at wavelength of 492 nm.

SDS-PAGE The method was described by Weber and Osborn[8]. The samples in loading buffer (0.5 g/L) were heated in boiling water for 5 min. Then it (15 µL) was added into the slot. The electrophoresis was carried out at 4 °C to minimize gel distortion. Each gel was run at an initial voltage of 90 V and a constant current of 20 mA for approximate 1 h until the bromophenol blue marker reached the end of the gel. The gel was stained, bleached, and then imaged in a gel Imaging system[9].

Effect of the pig ISPS homologue on cell proliferation Cells from various cell lines were incubated and tested against the purified pig ISPS homologue on cell proliferation. The method was similar to lymphocyte proliferation described above.

Statistical analysis Data were presented as mean±SD . Analysis of variance (ANOVA) was utilized for the comparison of several data sets to the control group. Data between both groups were compared by *t* test. *P*< 0.05 was considered to be significant.

RESULTS

Preparation of 2C4-McAb The monoclonal antibody 2C4-McAb was purified with Hitrap A column from affinity chromatography. There are two distinct protein elution curve peaks under UV 280 nm detector. The first large peak, eluted with PBS buffer (0.01 mol/L, pH 8.0) represents non-column binding multi-protein washouts. The second small peak, eluted with acetate buffer (0.1 mol/L, pH 3.0) represents monoclonal antibody (2C4-McAb). The separation of these two curves from affinity chromatography indicated that the preparation of 2C4-McAb was successful (Fig 1).

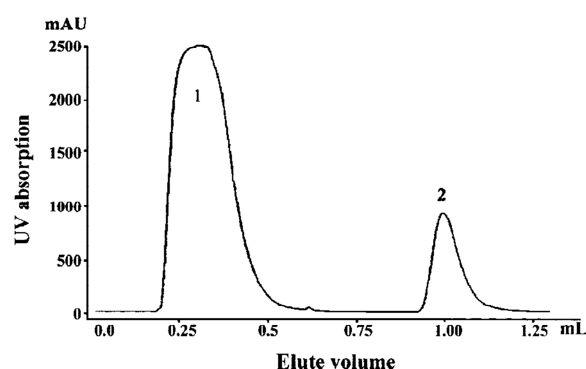

Fig 1. Purification of 2C4-McAb. 2C4-McAb was purified by Hitrap protein A column. Peak 1, multiprotein eluted with PBS (0.05 mol/L, pH 8.0); Peak 2, 2C4-McAb eluted with citric acid (0.1 mol/L, pH 3.0).

Identification of 2C4-McAb by SDS-PAGE The current 2C4-McAb preparation (Ab1) was tested by SDS-PAGE. We used standard protein marker (Low Molecular Weight, Calibration kit, Pharmacia Biotech) to estimate the molecular weight of 2C4-McAb and IgG (Sigma, G-7516) as the internal control. A previous lot of activity-tested 2C4-McAb from our laboratory (Ab2) was used as positive control. The SDS-PAGE electrophoresis demonstrated that the molecular weight of Ab1 and Ab2 are identical. When 2-mercaptoethanol was used, both Ab1 and Ab2 showed identical heavy chain (M_r 5000). These results from SDS-PAGE indicated that the current preparation of the 2C4-McAb was satisfactory (Fig 2).

Pig ISPS homologue purification by FPLC Affinity chromatography column coupled with 2C4-McAb was used. PBS (0.05 mol/L, pH 8.0) was used for non-binding protein removal and citric acid (0.1 mol/L, pH 3.0) was used for pig ISPS homologue-2C4-McAb antigen-antibody complex elution. UV 280 nm protein detector was used to monitor the chromatography process. At a flow rate of 0.5 mL/min, two distinct protein peaks were detected as shown in Fig 3.

Bioassay The inhibitory activity of the pig ISPS homologue was tested for Con A-induced lymphocyte proliferation. Purified pig ISPS homologue and the proteins from multi-protein peak were tested in serially di-

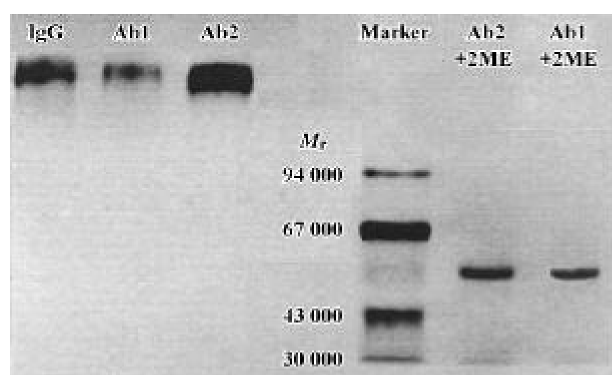

Fig 2. Identification of 2C4-McAb. 2C4-McAb was analyzed by SDS-PAGE. IgG, from SIGMA company; Ab1, 2C4-McAb prepared in the present experiment; Ab 2, 2C4-McAb prepared in the past.

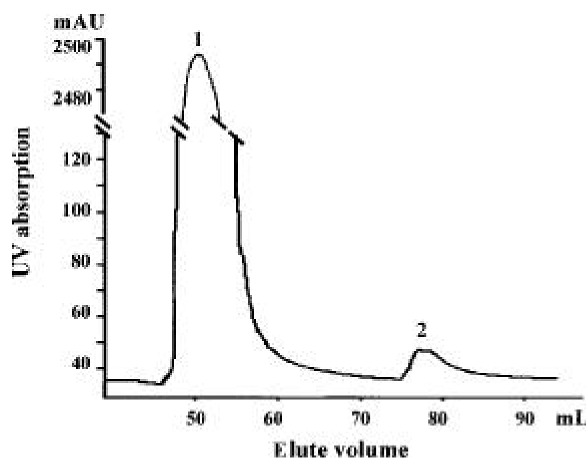

Fig 3. Purification of pig spleen extract by FPLC. Peak 1, multi-protein eluted with PBS (0.05 mol/L, pH 8.0); Peak 2, the purified protein eluted with citric acid (0.1 molL, pH 3.0)

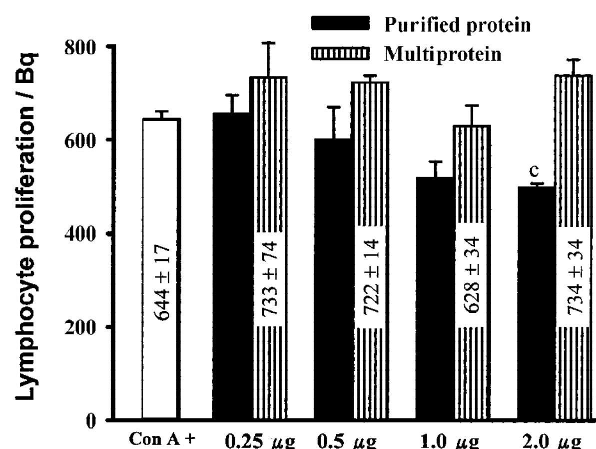

Fig 4. Suppressive effect of purified protein on lymphocyte proliferation. Con A+, lymphocyte proliferation induced by Con A (4 mg/L); Purified protein, culture medium containing Con A (4 mg/L) and various amount of purified protein. Multiprotein, culture medium containing Con A (4 mg/L) and the same amount of the multiprotein as that of purified protein. Mean±SD. ^c^*P*<0.01 *vs* multiprotein (ANOVA, *n*=6).

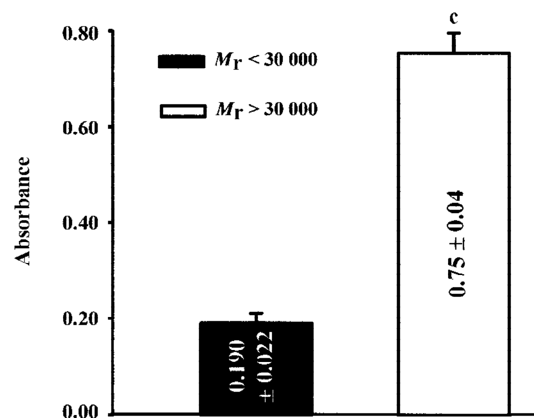

Fig 5. ELISA test of pig spleen purified protein. Mean± SD. ^c^*P*<0.01 *vs* protein with molecular weight less than 30 000 (*t* test, *n*=8).

luted doses (0.25, 0.5, 1, and 2 μg). Each dose was repeated in 3 identical wells of lymphocyte cultures and the Bq values (means±SD) of them were averaged. Con A (+) column was the internal positive control of normal lymphocyte proliferation (without adding any proteins) induced by Con A (4 mg/L). The results showed that the pig ISPS homologue significantly suppressed normal lymphocyte proliferation (Fig 4).

ELISA test Both pig spleen extraction ultrafiltration fractions (M_r>30 000 and <30 000) were tested with ELISA. Only the M_r >30 000 fraction showed binding affinity with 2C4-McAb, no binding is observed in the M_r<30 000 fraction (Fig 5).

Reversion test by 2C4-McAb Column 1 and 2 represent the lymphocyte proliferation without mitogen (Con A) stimulation (negative control) and with Con A (4 mg/L, positive control), respectively. The 2C4-McAb alone (column 3, 0.25 μg/well) had no significant effect on Con A-induced lymphocyte proliferation (column 2). However, Con A-induced lymphocyte proliferation was significantly suppressed by incubating the lymphocyte culture with pig ISPS homologue. This suppression could be partially reversed by the 2C4-McAb (Fig 6). These results suggested that the pig ISPS homologue was similar in antigenicity with mouse/rat ISPS and its function of lymphocyte proliferation suppression could be partially reversed by the 2C4-McAb.

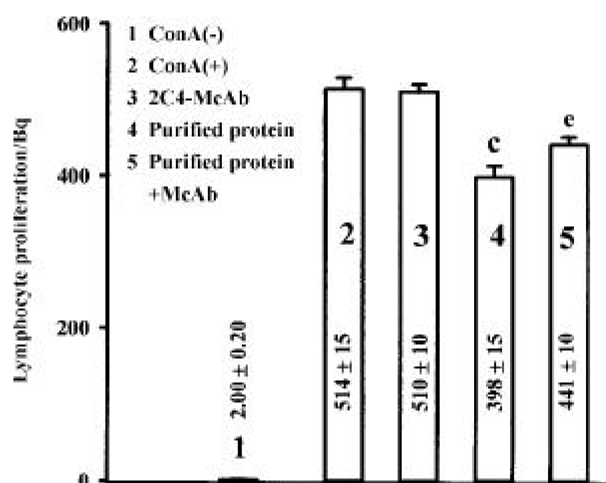

Fig 6. Reversed effect of 2C4-McAb on the suppression induced by pig spleen extract. Suppressive effect of the protein on lymphocyte proliferation was significantly reduced by adding 2C4-McAb. Mean±SD. [c]$P<0.01$ *vs* column 3. [e]$P<0.05$ *vs* column 4 (*t* test, $n=3$).

SDS-PAGE results SDS-PAGE electrophoresis results of the pig ISPS homologue were shown in Fig 7. The pig ISPS homologue treated with SDS had one prominent band (M_r 190 000). When it was treated with SDS and 2-mercaptoethanol, three bands with molecular weights of 68 000, 55 000, and 45 000 respectively were seen on the SDS-PAGE. This suggests that the pig ISPS homologue was probably composed of three subunits.

Selective suppressive effect of the protein on cell proliferation Six cell lines including U937 (macrophage), Mel (erythrocyte), 3T3 (fibroblast), Jurkat cell (T-cell), P810 (mast cell), and hepatocarcinoma were used for testing the effect of the protein on cell proliferation. In each experiment there were 3 experimental groups: blank group, control group, and experimental group. In blank group there was no protein to be added into the culture medium and the cell proliferation ([^{3}H]TdR uptake, Bq) was considered as 100 %. In control group (black column) and experimental group (white column) the protein (2 μg/well) from multiprotein peak and the pig ISPS homologue (2 μg/well) was added into cell culture medium respectively and their results of cell proliferation (Bq) were compared with the blank group. Proliferations of the cell line originating from immune cell such as U937, Jurkat cell, and P815 were found to be suppressed significantly. This suggested that the pig ISPS homologue had a selective proliferation suppressive activity on different cell lines (Fig 8).

DISCUSSION

The neuro-endocrine-immune system interaction influences a variety of physiological functions as well as pathological processes in mammals. There are many different components involved in immuno-regulatory mechanisms. The neuro-immune system has a very significant influence on the genesis, progression and prognosis of many human diseases. Many investigators have contributed to the discovery of immuno-regulation mechanisms. Alan TRAUTMANN and Eric VIVIER[10] proposed that the immune system and the nervous system shared a number of unique features.

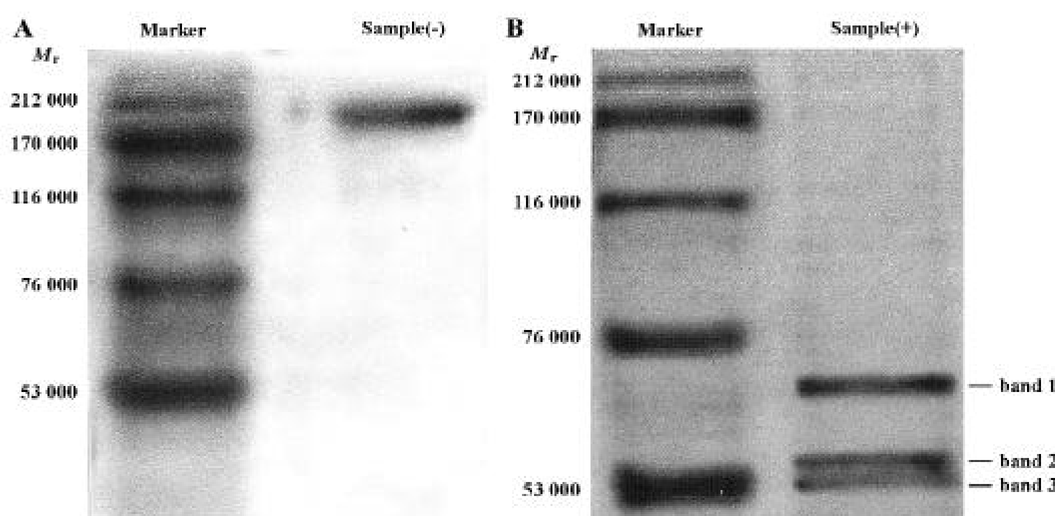

Fig 7. SDS-PAGE of purified sample. Sample(-) and sample (+) represent the sample treatment without and with 2-mercaptoethanol respectively in "A" and "B". Acrylamide concentration was 7 % for "A" and 10 % for "B".

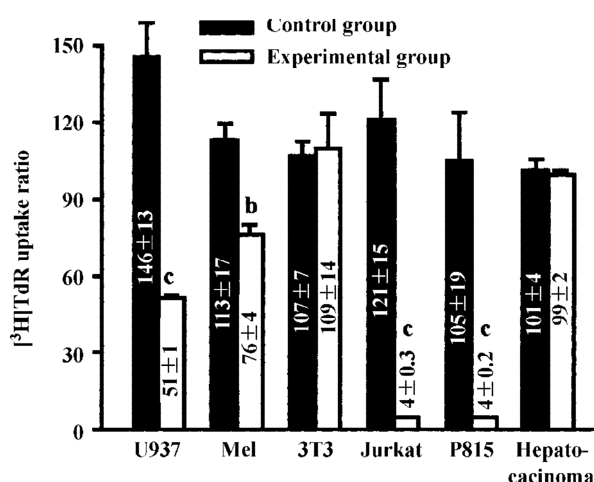

Fig 8. Selective suppression on cell proliferation induced by purified protein. Six cell lines including U937, Mel, 3T3, Jurkat cell, P 815, and hepatocarcinoma were used for testing the effect of the protein on cell proliferation. The first 5 cell lines are macrophage, erythrocyte, fibroblast, T- cell, and mast cell origin, respectively. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control (*t* test, *n*=3).

Both systems are composed of complex networks of primary and accessory cells that are in constant communication with each other. Although synapses in the nervous and immune systems are clearly different, they share a number of molecules for synapses formation and operation[10]. Khan and colleagues[11] reported that agrin, a well-characterized glycoprotein in neuromuscular junction(NMJ), which were specialized synapse between motor neurons and muscle cells, is also present in the immune system. They proposed that immune cell agrin might participate in the clustering of antigen-specific T-cell receptors (TCR) and the accessory co-stimulatory molecules between T-lymphocytes and antigen-presenting cells (APC). Agrin may also contribute to the establishment of neuronal and immunological memory[11].

Our present work showed that the pig ISPS homologue was found in pig spleen. Cell proliferations of the cell line originating from immune cell such as U937, Jurkat cell, and P815 were suppressed significantly by the pig ISPS homologue and the other cell line cells such as 3T3 (fibroblast) and hepatocarinoma cells were spared. Therefore, this protein has marked selectivity in participating immuno-activities. Our previous work showed with a molecular weight of 190 000, that ISPS was unlikely to belong to the well-known hormones, interleukins, and interferons families[12]. As first reported by our laboratory, the conditions under which ISPS is produced (in T-cell and controlled by CNS)[13,14] are quite unique. The simultaneous presence of both the immuno and neuro regulation is required. In conclusion, it is possible that ISPS belongs to a virgin protein family. It remains to be seen whether ISPS has any relationship to agrin.

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