J. Sci. I. R. Iran

Vol. 5 No. 3
Summer 1994

IN VITRO STUDY OF AN ENDOGENOUS IMMUNOSUPPRESSOR FACTOR DERIVED FROM HUMAN OR BOVINE SERUM

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Abstract

The effects of the human and bovine LSF (derived from sera) as well as their purified fractions were studied on murine lymphocytes reactions indicated by blast transformation (BT) assay, mixed lymphocyte culture (MLC) and IgG synthesis. The results indicated that bovine lipid suppressor factor (LSF) has significant immunosuppressive activity on lymphocytes proliferation both in BT and MLC assays. However, human LSF showed suppressive activity on lymphocyte proliferations only in MLC but not in BT assays. Concerning the effects of partially purified bovine LSF molecules on humoral immune response, we found that band #3 at 1:5 dilution strongly suppresses IgG synthesis of murine spleenocytes in response to pokeweed mitogen stimulation. Band #1 and band #3 are agents with cellular immunosuppressor activity. However, band #2 is not as suppressive as the other bands in BT, MLC, and IgG assays. It seems that the mixture of all bands together may have synergistic activity in the *in vitro* studies. This idea has been concluded from the results in BT and MLC assays which are presented in histograms #1 and #2. Before concluding that we have found an alternative immunotherapeutic agent to cyclosporine A, we need to study the molecular structure of these compounds as well as *in vivo* reactions of bovine LSF in skin transplantation. Since purified human LSF and its three bands did not indicate as much cellular immunosuppressive activity as bovine LSF, the study of their effects was not followed up in detail like bovine LSF in this project.

Introduction

One of the most important problems in organ transplantation in man and animals is rejection of graft by the recipient's immune system. At present, cyclosporine A (CsA) and azathioprine with corticosteroids are used to prevent or control graft rejection [1, 2], however nephrotoxicity and other side effects of these drugs limit their use [3]. Therefore, a

Keywords: Lipid Suppressor Factor [LSF]; Immunosuppressor; Human LSF; Endogenous LSF; Bovine LSF

safe immunosuppressor factor preferably endogenous factor is needed.

Kuo H. L. Hsu and his colleagues studied the immunosuppressive effect of lipoprotein separated from mice serum [4]. The results of these studies indicate that there are some endogenous immunosuppressive factors in the serum which prohibit proliferation of lymphocytes in cell culture even in the presence of mitogens. The *in vivo* studies by Kuo H. L. Hsu suggested that it may be possible to use the serum-derived nonspecific endogenous immunosuppressive factor for organ transplantation [5].

We have studied the effects of the human and bovine lipid suppressive factor (LSF) or their purified fractions on murine lymphocytes reactions indicated by blast transformation (BT) assay, mixed lymphocyte culture (MLC) and IgG synthesis. Our preliminary findings suggested bovine LSF has the potential to be used as an effective endogenous immunosuppressive factor in *in vitro* studies. We are planning to assess the effects of bovine LSF also in *in vivo* studies (on mice skin allografts) in order to speculate the possibility of using bovine LSF instead of CsA which is currently used in organ transplantation.

Materials and Methods

Extraction of LSF

Bovine serum was obtained from a Holstein cow. Human serum was collected from healthy volunteers. The collected sera were mixed with 40% ethanol that was precooled at -20°C. The mixture was centrifuged at 3000 rpm, 5°C, for 30 minutes, then the supernatant was removed. The pellet was mixed with chloroform/methanol (1:2) up to one third of the original serum volume. The mixture was filtered using No. 1 Whattman filter paper. The precipitated materials, named "lipid suppressor factor" (LSF), were separated using rotatory evaporator by several times of chloroform/methanol (1:2) extraction. The final extracts of human or bovine LSF were dissolved in chloroform/methanol (1:1) solvent and preserved at -20°C until use.

Purification of LSF

Partial purification of bovine and human LSF was accomplished using a thin layer chromatography (TLC) technique. Three bands were identified in TLC plates (250 micron thick silicone plates) after spraying with 40% sulfuric acid and baking at 85°C for 10 minutes. Bands #1, #2 and #3 LSF (both in human and bovine serum samples) were marked from the bottom to the top of TLC plates. The LSF bands were removed by scraping silicone off the TLC plates, then extracting by chloroform/methanol (1:1) solvent. The LSF bands were concentrated by a rotatory evaporator. Then the purified LSF bands were dissolved in 3-5 ml chloroform/methanol (1:1) solvent and were kept at -20°C for later use.

Cellular Immunity Assays

The biological activity of human or bovine crude LSF as well as the bovine LSF bands were separately evaluated using blast transformation assay, mixed lymphocyte culture and IgG synthesis.

Blast Transformation (BT) Assay

Eight to 12-week-old Balb/c mice were obtained from Charles River Laboratories, Inc. (Willington, MA, USA). Spleens were removed aseptically and placed in cold RPMI - 1640 cell culture media. A single cell suspension was made by teasing the spleen apart and then passing it through a 26-gauge needle. The cells were washed three times with cold medium RPMI - 1640 medium with 5% heat-inactivated fatal calf serum (FCS), 2mM/ml L-glutamine, 1000 units/ml penicillin and 100 microgram/ml streptomycine (complete medium). All the cultures were incubated at 37°C in a 5% CO_2 humidified incubator unless otherwise stated.

The lymphocyte transformation assay was performed as described by Khansari *et al.* [6]. Briefly, spleen cells were resuspended in complete medium to make a 10 million cell/ml cell suspension. One hundred microliters of the cell suspension were placed into each well of a 96-well flat bottom microtiter plate (quadriplicate). Each well received 100 microliters of medium containing either 0.8 microgram/ml phytohemagglutinin (PHA) or PHA plus various dilutions of LSF or PHA plus various concentrations of cyclosporine -A. Control culture wells received 100 microliters of the media only. Plates were incubated at 37°C, in a 5% CO_2 humidified incubator for 48 hours and then pulsed with 0.6 milicurie/well tritiated thymidine (3H-TdR). After incubation for an additional 18 hours, cells were harvested onto a fiber glass filter disk using an automatic cell harvester and scintillation liquid (Ecolume TM, 3 ml in each vial). Then radioactivity of the filter disks was measured by a beta counter (Beckman, bioanalysis system group, Arlington Height, IL., USA). The stimulation index (SI) was determined by the formula:

$$SI=\frac{\text{Mean CPM (stimulated cells)}}{\text{Mean CPM (control cells)}}$$

. The results were expressed in counts per minute (CPM).

Mixed Lymphocyte Culture (MLC)

Mixed lymphocyte culture was performed using the procedure described by Khansari *et al.* [7, 8]. Briefly, spleen cells from Balb/c mice were used as responder cells and spleen cells from an allogeneic mouse (C57BL/6) served as stimulator cells. Stimulator cells were treated with mitomycin-C to abolish proliferation. The responder and stimulator cells were resuspended (each separately) in RPMI 1640 medium containing 10% FCS to make a 10 million cell/ml cell suspension. Fifty microliters of the responder and 50 microliters of the stimulator cells were placed in each well of a 96-well flat bottomed microtiter plate. One hundred microliters of media were added to each well to bring the volume of each culture well to 200 microliters total. The control culture wells (background) received 50 microliters responder cells plus 150 microliters media only. The cultures were incubated at 37°C for 72 hours then pulsed with 0.6 mCi/well 3H-TdR and were then incubated for an additional 18 hours. At the end of the incubation period, cells were harvested onto a fiber glass filter disk and the radioactivity was measured by a beta counter as described above. The stimulation index (SI) was determined by the formula:

$$SI = \frac{\text{Mean CPM (responder + stimulator cells)}}{\text{Mean CPM (responder cells only)}}.$$ The results were expressed in counts per minute (CPM).

Immunoglobulin Synthesis

One hundred microliters of a cell suspension (10 million cells/ml), in complete medium containing 10% FCS, were placed in each well of a 24 - well microliter plate. Fifty microliters of medium containing pokeweed mitogen at 25 microgram/ml concentration was added to all wells, then 250 microliters of bovine LSF bands at different dilutions or CsA at different concentrations were added to the specified wells. The volume of each well was adjusted with complete media to contain 500 microliters/ml. The plates were incubated at 37°C, in a 5% CO_2 humidified incubator for 72 hours, then another 500 microliters of complete media was added to each well and the plates reincubated for a further three days. At the end of the incubation period, the culture supernatants were removed and the IgG content was determined by ELISA technique. Goat antimouse IgG, at two microgram/ml concentration, was used as coating buffer and goat antimouse IgG-ALP (alcaline phosphatase enzyme) was added in conjugated process for detecting IgG produced by lymphocytes in the cell cultures through measuring the observance values of samples, blanks, positive and negative control wells of ELISA microplates at 405 wave length. Mouse gama globulin at different concentrations was used as a positive control in order to design the standard curve of each serial ELISA technique. The results were expressed as nanograms per milliliter per one million cells, after converting the observance values of all samples on the standard curve, and were recorded in each experiment. IgG assays were performed three times, four replicates in each sample. The data was analyzed by using the one way analysis of variance.

Results and Discussion

The summary of results from adding partially purified bovine or human LSF to spleenocytes culture which were stimulated with PHA in BT assays is shown in Figure 1. Analysis of variance revealed a significant difference between five studied variables presented in Figure 1 ($P<0.05$). Since BT assays were performed three times, four replicates in each variable, each number on the bars of histogram #1 indicates the grand mean of 12 numbers expressed in CPM.

Partially bovine LSF, at 1:5 dilution, as well as cyclosporine A, at one microgram per milliliter concentration, showed a highly significant suppressive activity on murine lymphocytes in the presence of PHA mitogen at 0.8 microgram per ml concentration ($P<0.05$). Bovine LSF was more suppressive than human LSF (120 ± 16 CPM vs. 57670 ± 17 CPM) both at 1:5 dilution (Fig. 1). These results indicate that bovine not human LSF has significant immunosuppressive activity on lymphocyte proliferations.

Mixed lymphocytes culture is indicative of cytotoxic cells generation, a parameter of cell-mediated immunity [9-13]. We studied the effects of LSF on this response throughout, detecting the viability percentage of responder cells by using vital stain and microscopic observations after treating the stimulator cells with mitomycin-C. As is shown in Figure 2, both bovine and human LSF have suppressor activity on generation of cytotoxic cells. Analysis of variance revealed a significant difference between the five studied variables presented in Figure 2 ($P< 0.05$). Since MLC assays were performed three times, four replicates in each variable, each number on the bars of histogram #2 indicates the mean value of 12 numbers expressed in CPM. The suppressive activity of bovine LSF was significantly more than human LSF, both at 1:5 dilution (117 ± 34 CPM vs. 1611 + 32 CPM).

By purification of bovine LSF, using thin-layer chromatography (TLC) procedure, we were able to extract three different molecules having distinct

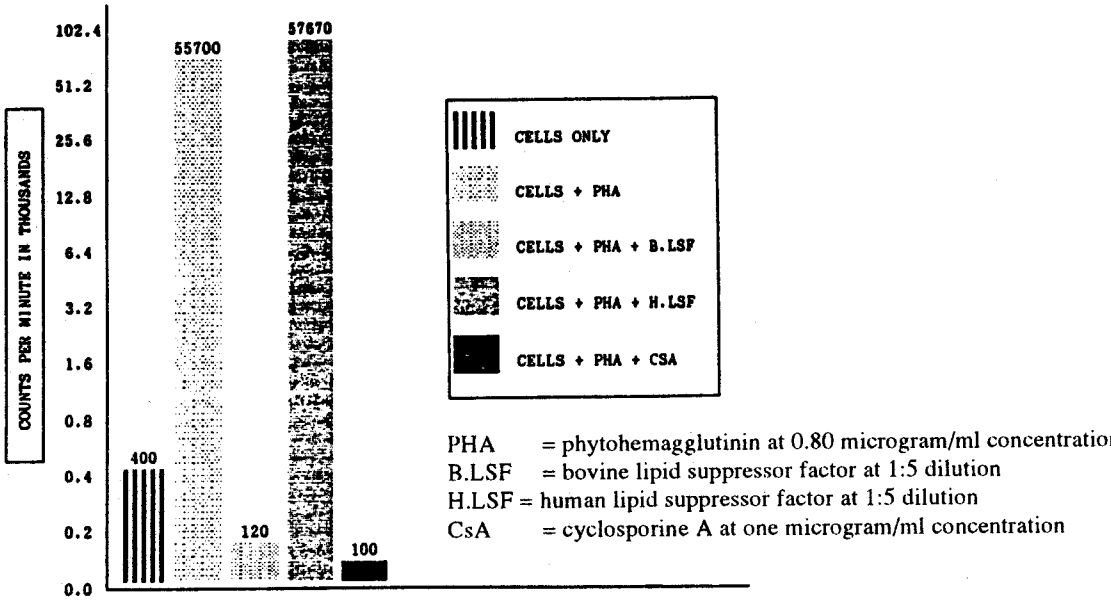

Figure 1. Effect of bovine and human "LSF" on murine lymphocyte blast transformation (BT) assay

Remarks

Analysis of variance revealed a significant difference between the five studied variables in Figure 1 ($P<0.05$).
Since BT assays were performed three times, four replicates in each variable, each number on the bars of histogram #1 indicates the mean value of 12 numbers obtained in three experiments expressed in counts per minute (CPM).

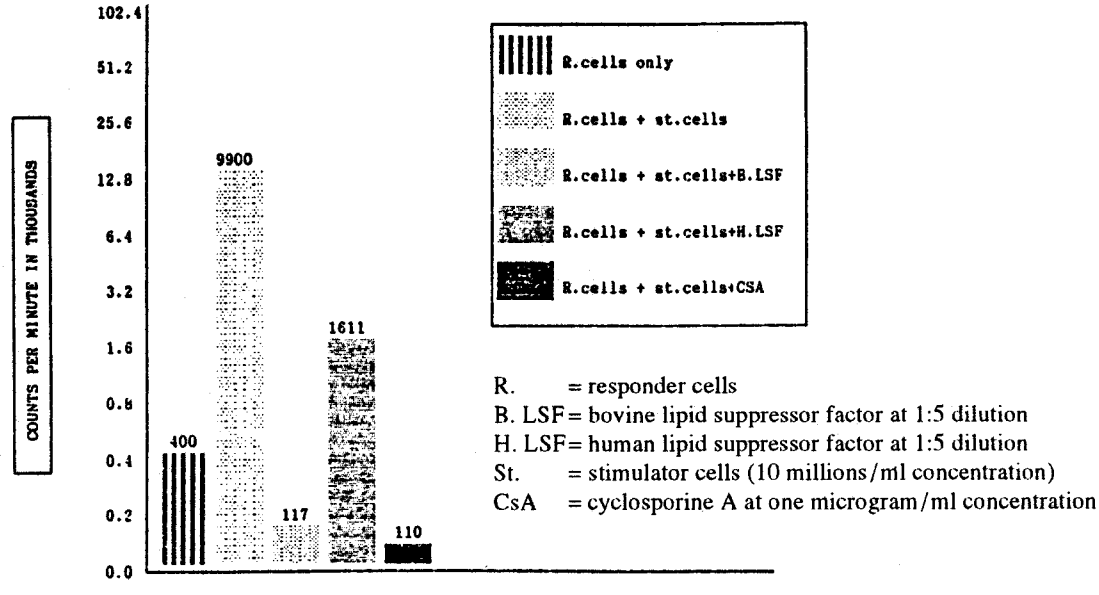

Figure 2. Effect of bovine and human LSF on murine mixed lymphocyte culture (MLC)

Remarks

Analysis of variance revealed a significant difference between the five studied variables in Figure 2 ($P<0.05$).
Since MLC assays were performed three times, four replicates in each variable, therefore each number on the bars of histogram #2 indicates the mean value of 12 numbers obtained in three experiments expressed in counts per minute (CPM).

relative mobility values. Both band #1 and band #3 (at 1:5 dilution) had significant suppressor activity on spleenocyte blast transformation in response to PHA at 0.8 microgram/ml concentration (compare the results in Figure 3). However, the suppressive activity of band #1 (1774 ± 32 CPM) and band #3 (1840±90 CPM) was less than cyclosporine A at one microgram/ml concentration (158 ± 16 CPM). As is shown in Figure 3, the suppressive activity of band #2 (37640±23 CPM) was significantly less than band #1 and band #3 at the same dilution ($P<0.05$).

The suppressor activity of different bands on mixed lymphocyte culture (MLC) is shown in Figure 4. This data indicates that band #1 (223±23 CPM) had higher suppressor activity than the other two bands and band #3 (486 ± 131 CPM) had higher suppressor activity than band #2 (11667 ± 16 CPM). Analysis of variance revealed a significant difference between the six studied variables presented in Figure 4 ($P<0.05$).

Finally, we studied the effect of partially purified bovine LSF molecules at different dilutions on humoral immune response using *in vitro* IgG synthesis assays. Results are shown in Figures 5 and 6. In these experiments we found that band #3 at 1:5 dilution suppresses IgG synthesis of murine spleenocytes in response to pokeweed mitogen stimulation, compared to the other bands. However CsA at 1 microgram/ml concentration was more suppressive than band #3 at 1:5 dilution (Fig. 5). The mean value of IgG production, measured by ELISA technique in the presence of band #3 of bovine LSF at 1:5 dilution and pokeweed mitogen at 25 microgram/ml concentration, was 8.00 ng/ml/one million cells which was significantly less than the mean value of IgG produced in the presence of pokeweed mitogen alone (24 ng/ml/one million cells) at the same concentration. These results, which are presented in Figure 5, indicate that the suppressive activity of band #3 was significantly higher than bands #1 and #2 but lower than CsA at one microgram concentration/ml.

A similar situation was observed in another experiment, the results of which are shown in Figure 6. In this experiment, the three bands of bovine LSF at 1:10 dilution were compared with CsA at three microgram/ml concentration as well as with cells plus pokeweed mitogen alone. The mean value of IgG produced by murine lymphocytes in the presence of band #3 was 8.30 ng/ml/one million cells whereas this value was significantly less than band #2 (25.7 ng/ml/one million cells) and even band # 1 (11.70 ng/ml/one million cells) as well as in the presence of pokeweed mitogen alone which was 24 ng/ml/one million cells.

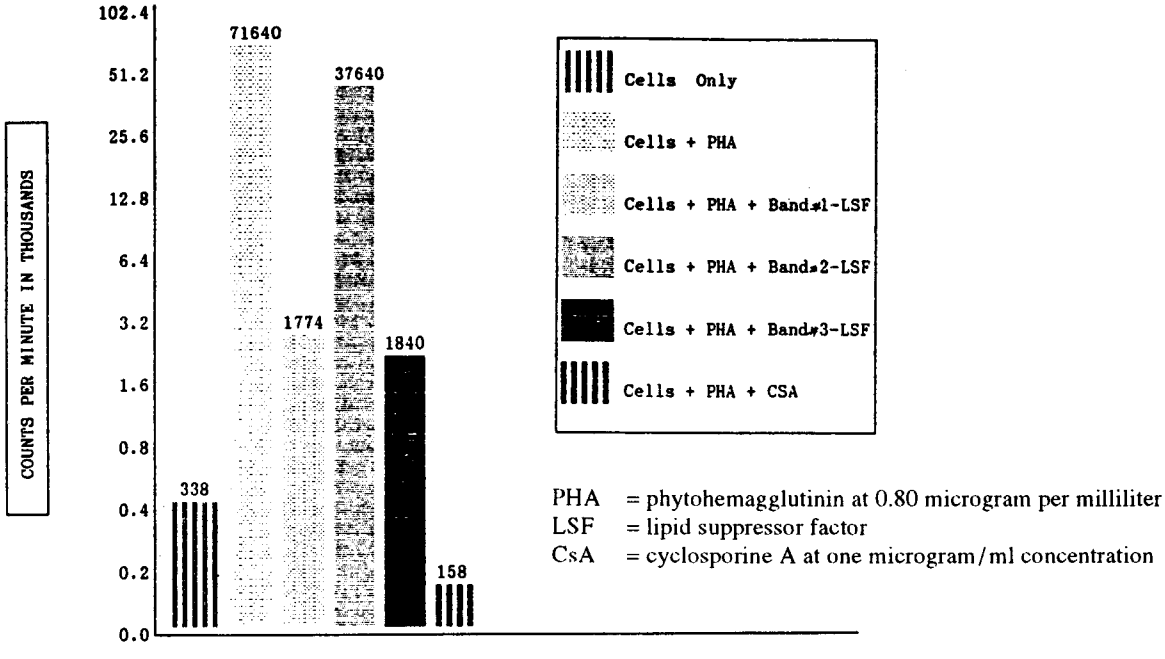

Figure 3. Effect of partially purified bovine LSF (bands #1, #2, & #3) on murine lymphocyte blast transformation (BT) assay

Remarks

Analysis of variance revealed a significant difference between the six studied variables in Figure 3 ($P<0.05$).

BT assays were performed three times (four replicates in each variable), therefore each number on the bars of histogram #3 indicates the mean value of 12 numbers obtained in experiments expressed in counts per minute (CPM).

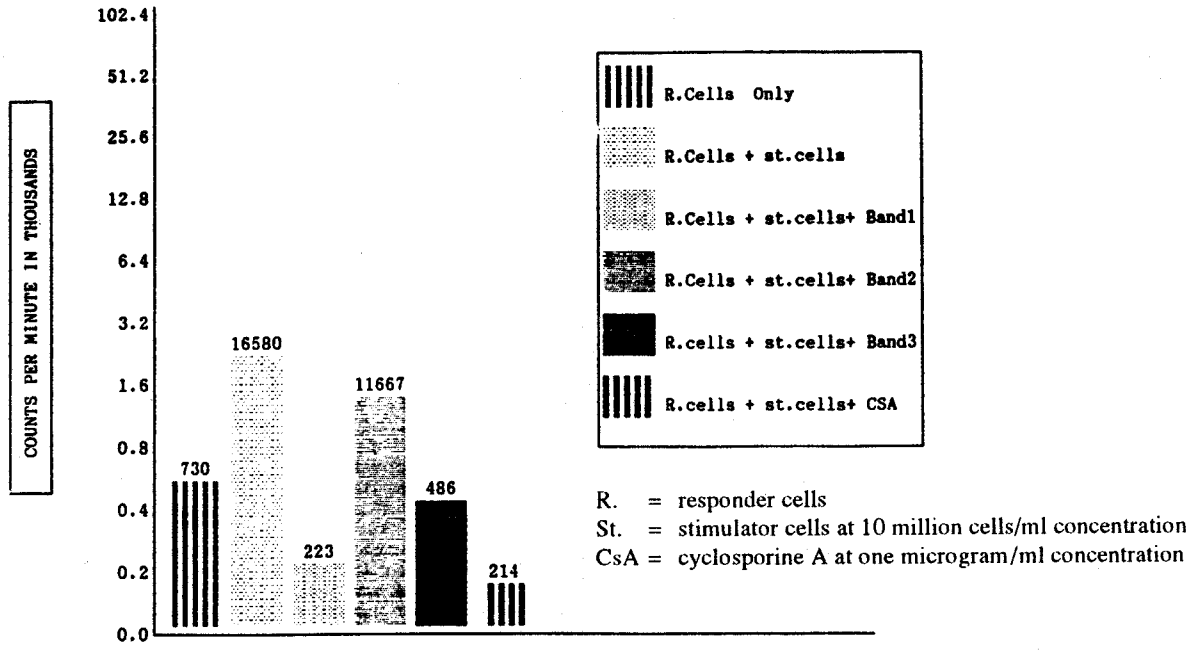

Figure 4. Effect of partially purified bovine LSF (bands #1, #2 & #3) on murine mixed lymphocyte culture (MLC)

Remarks

Bands #1, #2, #3 = bovine LSF bands at 1:5 dilution

Analysis of variance revealed a significant difference between the six studied variables in Figure 4 ($P<0.05$). MLC assays were performed three times, four replicates in each variable, therefore each number on the bars of histogram #4 represents the mean value of 12 numbers obtained in three experiments expressed in counts per minute (CPM).

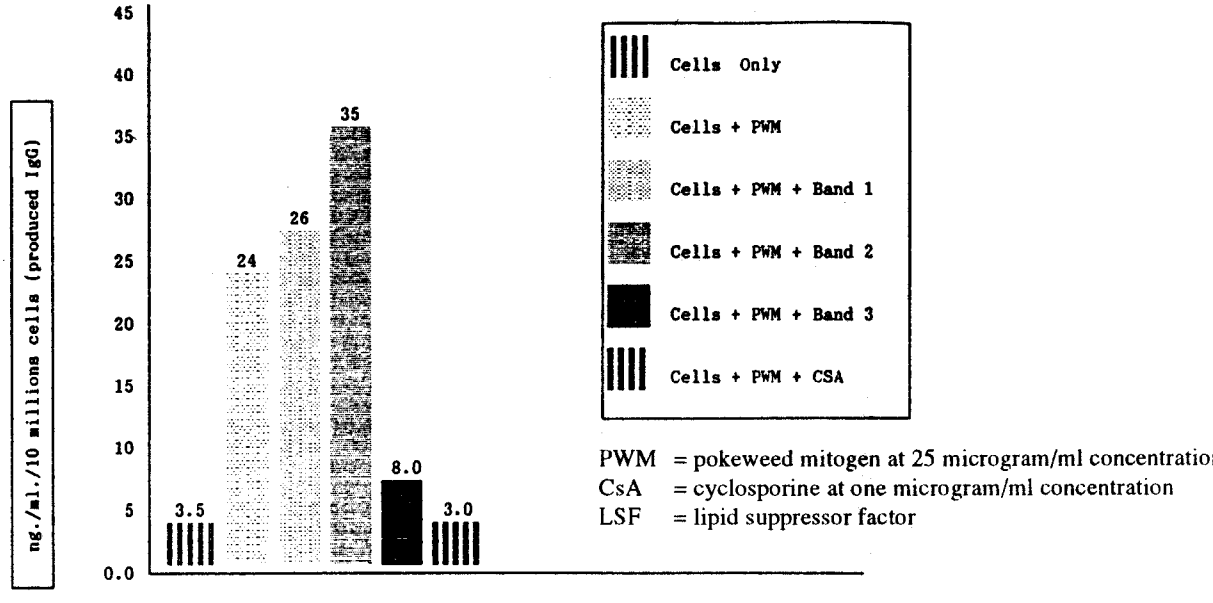

Figure 5. Effect of partially purified bovine LSF (bands #1, #2 & #3) at 1:5 dilution on murine immunoglobulin producer lymphocyte (IgG synthesis), measured by ELISA technique, expressed in nanogram per milliliter per one million cells

Remarks

Analysis of variance revealed a significant difference between the six studied varibles in Figure 5 ($P<0.05$).

Since IgG assays were performed three times, four replicates in each variable, each number on the bars of histogram #5 represents the mean value of 12 numbers obtained in three experiments expressed in nanograms per milliliter/one million cells.

The suppressive activity of CsA at three microgram/ml concentration in all IgG assays was significantly more than the three bands of bovine LSF as presented in Figure 6. Therefore, in an attempt to introduce an endogenous immunosuppressive agent at the level of cell culture (*in vitro* study), we found that there are at least two molecules in bovine serum that appear to be lipid in nature and suppress both humoral and cellular immunity. These molecules were called band #3 and band #1, respectively, which were detected by TLC technique and purified by several evaporations of extracts and preserved in chloroform/methanol (1:1) solution.

Cellular immunity can be evaluated by various assays [18, 19]. The most popular ones are blast transformation and cytotoxic generation. We studied the effect of LSF on both assays and found that the crude material would strongly suppress both functions (Figs. 1 & 2).

After extraction with chloroform / methanol, in blast transformation, human LSF did not suppress lymphocyte proliferation which in MLC assay it did. This phenomenon suggests that these molecules are functionally specific and have an active role in the body. After partial purification of the molecules, we found that the purified preparation has less activity in general. Additionally, not all bands have the same activity (Figs. 3. & 4). Having less activity in comparison with crude material (LSF before purification) suggests that perhaps a mixture of all the three bands together may have synergistic activity, although band #2 did not show any suppressive activity in either assays of IgG synthesis.

When we studied the effect of purified LSF on IgG synthesis, only band #3 revealed consistent suppressor activity (Fig. 5). This is a very interesting phenomenon because it suggests that this molecule might have certain specific regulatory functions and that we may have found two different immunosuppressive agents from bovine serum: band #1, an agent with cellular immunosuppressor and band #3 a humoral immunosuppressor. If this is the case, this is the first time that such specific immunosuppressive compounds have been introduced which might have potential applications for various immunosuppressive conditions both in cellular and humoral immunity reactions, respectively, and possibly for various immunotherapeutic conditions such as organ transplantations.

In human patients, cyclosporine nephrotoxicity is

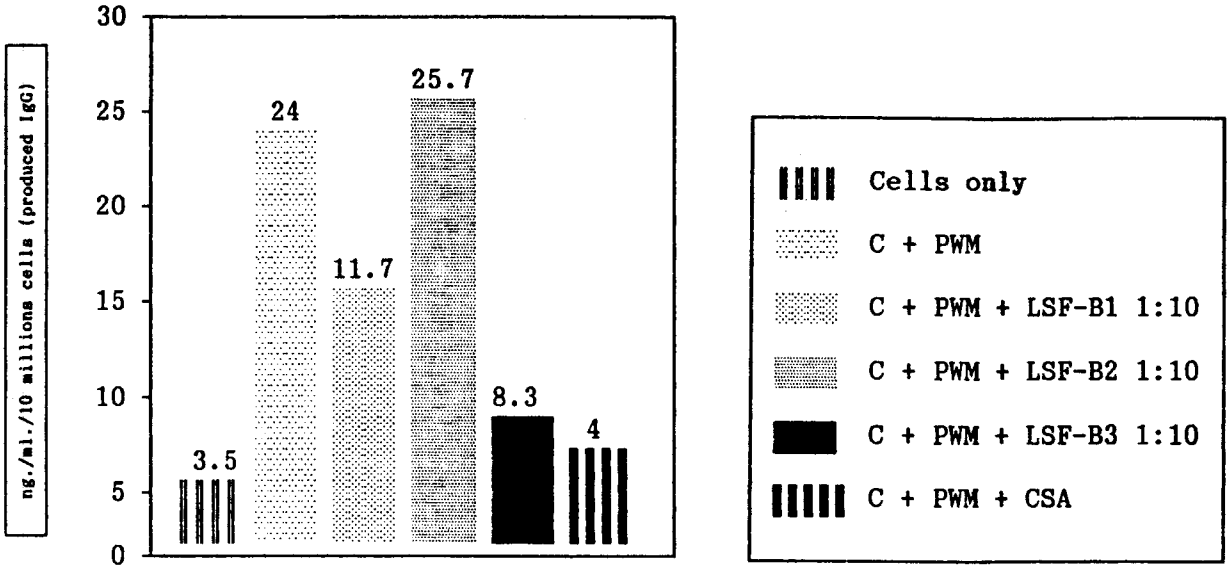

Figure 6. Comparative immunosuppressive effects of bovine LSF bands (#1, #2 & #3) at 1:10 dilution and CsA at 3 microgram/ml concentration on murine immunoglobulin producer lymphocyte (IgG synthesis), measured by ELISA thechnique.

Remarks

C = cells
PWM = pokeweed mitogen at 25 microgram/ml concentration
CsA = cyclosporine A
B1, B2, B3 = bands #1, #2 and #3 bovine LSF
LSF = lipid suppressor factor

its major limiting factor as an immunosuppresive drug. Several studies indicate that cyclosporine can permit the development of lethal bacterial and fungal infection [22]. Cyclosporine is generally not nephrotoxic in dogs and cats. Hepatotoxicity also occurs in human patients but has not been a problem in dogs and cats. In general, the suppressive activity of bovine LSF is comparable to that of cyclosporine A, a potent immunosuppressive agent used in autoimmune therapy and for prevention of graft rejection [14, 18, 22].

The effect of band #3 bovine LSF at 1:10 dilution is specifically comparable to the effect of cyclosporine A at three microgram per milliliter concentration on murine immunoglobulin producer lymphocytes concerning their suppressive activities, as presented in Figure 6. However, in order to clarify and confirm this hypothesis, we need to study the molecular structure of these compounds as well as their mechanisms of action and also the origin of these molecules before concluding that we have found an alternative immunotherapeutic agent to CsA which is used in organ transplantation as an immunosuppressive drug.

Acknowledgements

This research project was carried out in the Immunology Laboratory at the Department of Veterinary Science and Microbiology, NDSU, through program # P-I-3356 and under the supervision of Dr. Nematollah Khansari, Professor of Immunology, during the author's period of sabbatical leave at the North Dakota State University. The cooperation of Dr. Iraj Norozian, Associate Professor at Tehran University in statistical analysis is appreciated. The author was also financially supported by the University of Tehran and the Ministry of Culture and Higher Education of the Islamic Republic of Iran. The author would like to express his appreciation to Professor Herbert Smith, head of Department of Veterinary Science and Microbiology, NDSU, as well as Mr. Sandra Halbrook, the officer of the International Exchange Scientists Office and Thomas Gustad for his technical assistance in the Immunology Laboratory at NDSU, USA.

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