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THE INFLUENCE OF BOVINE SEMINAL PLASMA FRACTIONS OBTAINED BY DEAE-SEPHACEL CHROMATOGRAPHY ON BOVINE LYMPHOCYTE BLASTOGENESIS

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The aim of this study was to separate protein molecules from bovine seminal plasma and to investigate their influence on mitogen induced bovine lymphocyte blastogenesis.

Pooled bovine seminal plasma was chromatographed on an ion exchange column (DEAE-Sephacel) and six fractions were obtained. Their influence on bovine lymphocyte blastogenesis was studied in vitro in cell cultures stimulated with suboptimal doses of PHA and Con A. Protein molecules that were present in fractions 1 and 6 were inhibitory in PHA stimulated cultures but only those from fraction 1 if cell proliferation was induced by Con A.

We were also able to demonstrate that in cell cultures stimulated with PHA protein molecules from fractions 2 and 4 had stimulative effects. When bovine lymphocyte blastogenesis was induced with Con A only molecules from fraction 4 stimulated cell proliferation.

In all our tests pooled seminal plasma exerted very strong inhibitory effects on mitogen induced bovine lymphocyte blastogenesis.

Key words: bovine seminal plasma, immuno-suppression, lymphocytes

INTRODUCTION

Bovine seminal plasma (BSP) is an extremely complex fluid whose constituents have an important role in the migration and survival of sperm in the female genital tract. At the same time sperm and seminal antigens may provoke an immune response in heterologous species but they are rarely auto or isoantigenic (Fahmi et al. 1985)

One of the possible explanations why the antibody-synthesizing system in cows might not be activated is seen in the data of Prakash et al. (1976), Lugaro et al. (1984), and Fahmi et al. (1985, 1985a). They reported that BSP contains inhibitors of lymphocyte functions when tested in vitro. Similar data

have been reported for human seminal plasma (Stites and Erickson 1975, Lord et al. 1977, Marcus et al. 1978, Franken and Slabber 1981, Quayle et al. 1987) as well as for some laboratory animal species (Anderson and Tarter 1982). Prakash and colleagues (1976) found that an inhibitory factor from BSP (named seminal plasmin), isolated after chromatography on a Sephadex G-100 column had a molecular weight above 100 kDa and that one of the protein fractions was stimulative. Lugaro et al. (1984) isolated from BSP a low molecular weight factor (800-1000 Da) that inhibited RNA synthesis. In 1985. Fahmi et al, reported that immunosuppressive activity of BSP is associated with seminal plasma proteins of 150 kDa and also with those of MW less than 50 kDa. They suggested that interaction of several low molecular components might be necessary for full expression of inhibitory activity. Matoušek and his colleagues proposed (results reviewed by Matoušek 1985.) that an enzyme – AS RNase, originating from the seminal vesicles of bulls, is responsible for the embryotoxic, cancerostatic and immunosuppressive effects of BSP. Immunosuppressive effects of AS RNase were later confirmed by Tamburrini et al. (1990). In another study a basic protein distinct from RNase was isolated from BSP and exerted inhibitory effects on Con A induced bovine lymphocyte proliferation (Derwenskus et al. 1989). Using the direct immunofluorescent method Vukotić and Pavlović (1981) showed that component(s) from BSP can bind to chromatin material of a wide variety of cell types.

The objective of this work was to separate protein molecules from BSP on a DEAE-Sephacel column and to investigate their influence on bovine lymphocyte blastogenesis.

MATERIAL AND METHODS

Preparation of seminal plasma. Semen was collected from black and white spotted bulls (Holstein breed) by an artifical vagina and only samples that possessed normal characteristics of motility, morphology and concentration were used in the experiments. The ejaculates were cooled at+4^{0}C over 3 hrs and seminal plasma was separated by three sequential centrifugations of 15 min. at 400, 800 and 1200 g at +4^{0}C to minimize the acrosome damage. Samples were kept frozen at -20 ^{0}C until used.

Chromatography. A pool of BSP was created by mixing equal volumes (1 ml) of seminal plasma from the ten bulls. Pooled seminal plasma (10 ml) was diluted in 3 mM phosphate buffered saline PBS up to 80 ml and chromatographed on a DEAE-Sephacel column (26 x 80 mm; Pharmacia), previously equilibrated with the same buffer. Fractions (10 ml) were eluted by a linear gradient up to 0,4 M NaCl in 3 mM phosphate buffered saline (200 + 200 ml) and collected using an automatic collector (Multirac 2111; (LKB-Bromma, Sweden). The elution curve was recorded on a spectrophotometer (Uvicord 2138; LKB – Bromma, Sweden) at 278 nm. According to the elution curve, fractions were pooled into six groups and concentrated using an Amicon-Diaflo membrane PM 30 (cut off 30 kDa). Protein concentration in seminal plasma fractions was determined using BCA-assay (Sigma No TPRO – 562). Before

testing in cell cultures BSP and its fractions were dialyzed against PBS (pH 7,2) and RPMI 1640 (Gibco) basic solution (1:1000) at +4^{0}C over 24hrs. Afterwards they were diluted with final RPMI 1640 medium[1] to achieve a final concentration of 1%.

Bovine lymphocyte blastogenesis (BLB). The bovine lymphocyte blastogenesis test was similar to that developed by Muscoplat et al. (1974) and was described in detail earlier (Lazarevic et al. 1992). Mononuclear cells were obtained from healthy, nongravid black and white spotted cows. Cells (2 x 10^5/well) were stimulated with 5 and 10 μg/ml of PHA (Bacto-Phytohemagglutinin, DIFCO) and 2,5 and 5 μug/ml of Con A (Pharmacia) and incubated over 72 hrs in flat-bottomed 96-well microtitre plates (Costar, Cambridge). They were pulsed with 1 μCi of ^{3}H-thymidine/well 18 hrs prior to harvesting and the influence of BSP and its fractions was measured as a percent of ^{3}H-thymidine incorporation compared to the controls. For cytotoxicity testing, cells were incubated with BSP and its fractions without mitogen.

Antigenic analysis. IFF characterization of protein molecules in the active fractions of seminal plasma was prepared by Schreideggers method (1955) using polyspecific anti-BSP and anti-BS (bovine serum) sera obtained by immunization of rabbits.

RESULTS

The results obtained in these experiments are summarized in Figures 1 and 2. Seminal plasma fractions were tested in a final concentration of 1% because pooled BSP exerts strong inhibition when tested in the same concentration. This inhibition is shown on the right side of each graph.

All tests were performed in triplicate and mean values and standard errors were calculated. No cytotoxicity effects were observed due to the dialysis procedure (nondialysed BSP is cytotoxic in low dilution, Fahmi et al. 1985.)

Fractions 1 and 6 inhibited lymphocyte proliferation if the cells were stimulated with PHA, while fraction 4 and to a certain extent fraction 2 were both stimulative. These effects were markedly expressed when suboptimal doses of mitogen were used (5 μg/ml) to stimulate cell proliferation (Figure 1).

Similar results were obtained when a higher concentration (10 μg/ml) of PHA was used to stimulate cell proliferation (data not shown here).

In Con A stimulated cell cultures only fraction 1 had an inhibitory effect on bovine lymphocyte blastogenesis, while fraction 4 stimulated cell proliferation (Figure 2.) Both inhibition and stimulation were higher with suboptimal (2,5 μg/ml) doses of mitogen. Similar results were obtained when a higher concentration (5 μg/ml) of Con A was used to stimulate cell proliferation (data not shown here).

[1]RPMI 1640 basic solution pH 7,2
RPMI (Gibco) 5,2175 g
H_2O redest. 380 ml
$NaHCO_3$ (2,8%) 35,7 ml
H_2O redist. ad 480 ml
pH was adjusted with 0,1 M HCl or NaOH

RPMI final medium
Hepes (Flow 20 mM) 2 ml
FCS (Flow) 5 ml
Streptomycin (Galenika) 0,1 g
RPMI 1640 basic sol. ad 100 ml
Nystatin 0,01 ml

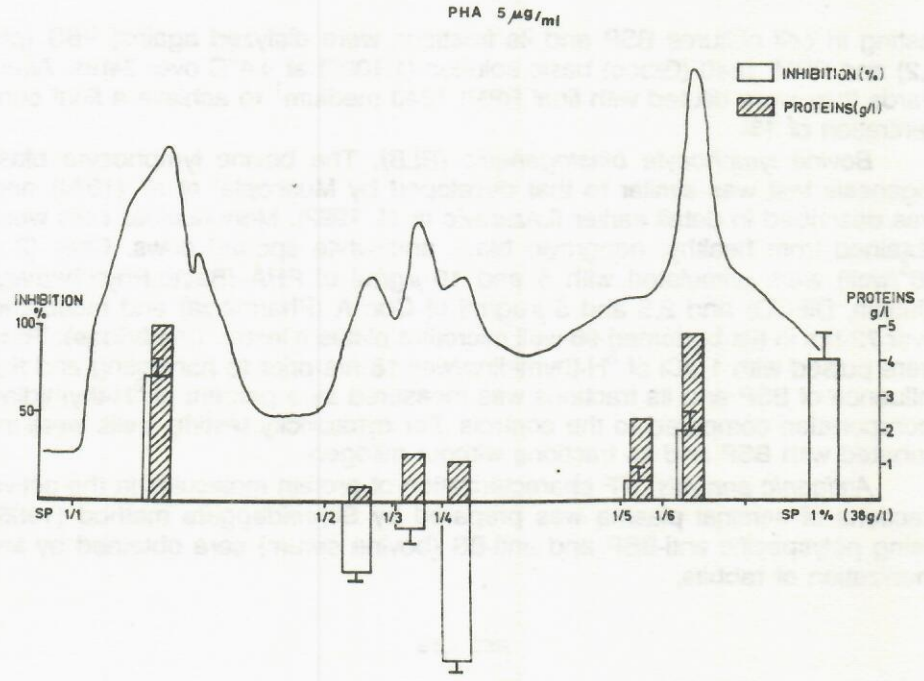

Figure 1. The infuence (% of inhibition) of BSP fractions on bovine lymphocytes stimulated with 5 μg/ml *op* IIHA

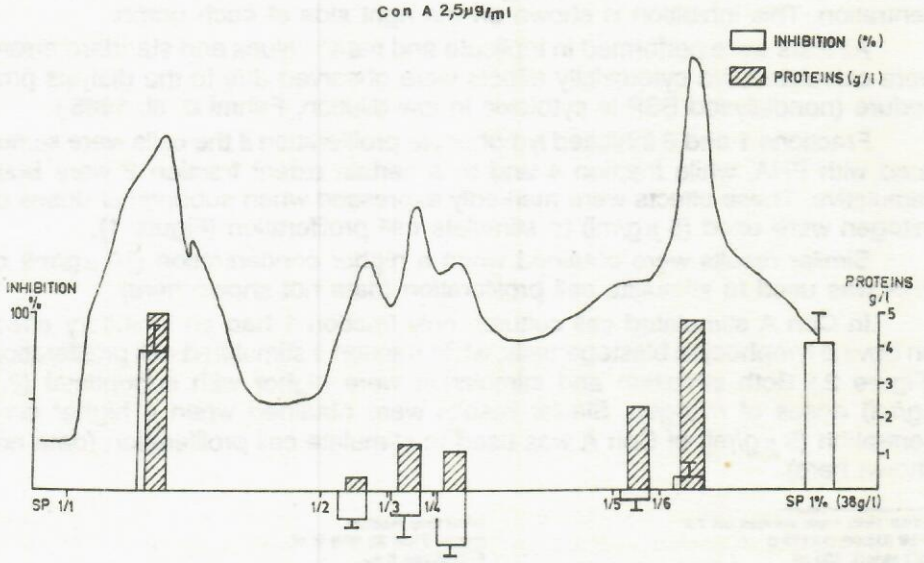

Figure 2. The influence (% of inhibition) of BSP fraction on bovine lymphocytes stimulated with 2.5 μg/ml of Con A

The degree of BLB inhibition did not correlate with the amount of total protein in the fractions.

IEF analyses of protein molecules that were present in the active fractions of BSP, with polyspecific antisera against bovine seminal plasma. (1,2,4 and 6) also revealed the presence of different protein molecules separated on the basis of their different electric charge (data not shown here).

DISCUSSION

Bovine seminal plasma contains a wide variety of proteins and it seemed reasonable that chromatography on a ion-exchange column (DEAE-Sephacel) might have an advantage compared to simple gel-filtration. This approach proved to be satisfactory because BSP was clearly separated into six fractions exerting completely different biological effects (from strong inhibition to strong stimulation of lymphocyte blastogenesis). Using Sephadex columns it was possible to separate seminal plasma proteins in 5 fractions (Prakash et al. 1976, Fahmi et al. 1985 a). Our results demonstrated that active (stimulative or inhibiting) factors from BSP do not differ only by molecular mass but also by other physico-chemical properties i. e. charge, resulting in a better separation on anion-exchange column. It is our opinion that both inhibitory or stimulative effects are rather characteristic of single factors than of combinations as previously suggested (Fahmi et al. 1985a). In our experiments the influence of seminal plasma fractions was different in PHA and Con A stimulated cell cultures. When bovine mononuclear cells were stimulated with PHA, fractions 1, 2, 4 and 6 were active but only fractions 1 and 6 were active when stimulation was induced with Con A. This can lead us to the conclusion that the mechanisms of inhibition or stimulation are not the same for both mitogens.

Although the influence of pooled bovine seminal plasma on mitogen induced bovine lymphocyte blastogenesis was inhibitory in all experiments, our finding that certain fractions exerted stimulative effects is not insignificant. Only from data presented by Prakash et al. (1976) was it possible to realize that some seminal plasma constituents exerted stimulative effects on mitogen induced lymphocyte proliferation in vitro and generally much more attention has been paid to inhibitory substances, bearing in mind that nonfractionated seminal plasma suppresses lymphocyte proliferation. In the cases of oligo and azoospermia in man, it was documented (Gershbein and Theilen 1988.) that the protein composition of seminal plasma might be changed, thus resulting in changes of their biological function. The authors pointed out that of the amount of total protein was nearly the same in seminal plasma samples derived from oligo and normospermatic ejaculates but the albumin concentration in normospermatic material was significantly higher. On the contrary Stanislavov et al. (1978) did not find significant differences in the protein profile of seminal plasma of males with disturbed fertility but their study did not include quantitative methods. We have recently found that the amount of total protein in bovine seminal plasma samples derived from oligospermatic ejaculates was significantly higher

in comparison to normospermatic ones (Lazarević, 1994. unpublished results) In our opinion this problem deserves more attention.

Therefore, it might be of interest to investigate the influence of seminal plasma derived from ejaculates of poor quality on mitogen induced lymphocyte blastogenesis and to correlate these results with their protein composition. This approach can be very useful in studies of some infertility cases., especially in humans.

We may, thus, conclude that bovine seminal plasma contains protein molecules of different categories with both inhibitory and stimulative effects on mitogen induced bovine lymphocyte blastogenesis. These molecules exerted different influences on PHA and Con A stimulated bovine lymphocites and their precise characterization will be the objective of our future investigations.

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UTICAJ FRAKCIJA SEMENE PLAZME BIKA DOBIJENIH HROMATOGRAFIJOM NA KOLONI DEAE – SEPHACEL-A NA PROLIFERATIVNI ODGOVOR GOVEĐIH LIMFOCITA

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SADRŽAJ

Cilj ovih istraživanja je bio da se izvrši separacija proteinskih molekula semene plazme bika i ispita uticaj dobijenih frakcija na mitogenima indukovanu proliferaciju govođih limfocita.

Hromatografskom separacijom zbirnog uzorka semene plazme bika na koloni anjonskog izmenjivača (DEAE-Sephacel) dobijeno je šest frakcija i ispitan je njihov uticaj na proliferativni odgovor govođih limfocita stimulisanih suboptimalnim dozama mitogena PHA i Con A. Molekuli prisutni u frakcijama 1 i 6 dovodili su do inhibicije proliferativnog odgovora govođih limfocita u ćelijskim kulturama stimulisanim sa PHA dok je pri stimulaciji sa Con A samo prva frakcija ispoljavala supresivno delovanje.

Istovremeno je uočeno da proteinski molekuli prisutni u frakcijama 2 i 4 dovode do stimulacije proliferativnog odgovora u ćelijskim kulturama stimulisanim sa PHA dok je pri stimulaciji sa Con A samo frakcija 4 delovala stimulatorno.

U svim našim ogledima, delovanje nefrakcionisane semene plazme na proliferativni odgovor govođih limfocita bilo je izrazito inhibitorno.