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Seminal plasma suppression of human lymphocyte responses *in vitro* requires the presence of bovine serum factors

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SUMMARY

The effect of bovine sera on the ability of human seminal plasma (SP) to suppress lymphocyte responses was investigated. Marked suppression of natural cell-mediated cytotoxicity (NCMC) against K562 targets was observed when effectors were pretreated for 1 h with SP (1 : 50 dilution) in the presence of 10% newborn calf serum (NBCS). Some suppression of natural cytotoxicity was observed when the effectors were treated with SP in the presence of 10% fetal calf serum (FCS) and this suppression was greater if the length of pretreatment with SP was increased to 20 h. Suppression of NCMC did not occur, or was considerably less, when the effectors were treated with SP in the presence of 10% autologous human plasma or in HB103 serum-free medium. The effect of bovine sera on suppression of T lymphocyte response to mitogen was also examined. Pre-treatment of lymphocytes with SP (1 : 50 dilution) for 1 h in the presence of 10% NBCS was sufficient to abrogate completely the stimulatory effect of PHA. In the presence of 10% FCS it was necessary for SP to be present throughout the assay for suppresion to occur. In HB103 medium, stimulation was only slightly decreased below control values when SP was present throughout the assay, but suppression was considerably less than that obtained upon addition of NBCS or FCS to the culture medium. These findings imply that suppression of lymphocyte activity by SP is dependent on the presence of exogenous serum co-factors and in the light of this finding, the clinical relevance of SP suppression may require re-examination.

Keywords seminal plasma natural killer cells T cell mitogenesis bovine serum

INTRODUCTION

Many reports have demonstrated the ability of seminal plasma (SP) to inhibit the functional activity of lymphocytes. The majority of these studies show SP or a fraction of SP to suppress the in-vitro lymphocyte response to T or B cell mitogens (Stites & Erickson, 1975; Lord, Sensabaugh & Stites, 1977; Marcus *et al.*, 1978; Franken & Slabber, 1981; Majumdar *et al.*, 1982). In addition, SP also inhibits the primary and secondary antibody response when injected into mice (Anderson & Tarter, 1982), complement activity (Petersen *et al.*, 1980) and macrophage function and mobility (Stankova *et al.*, 1976). Recently SP has been shown to inhibit lymphocyte-mediated natural cytotoxicity (James & Szymaniec, 1985) and also interferon (IFN), natural killer cell activity (Rees *et al.*, 1986).

Many biologically active molecules present in SP have been proposed as mediators of

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suppression (James & Hargreave 1984), although as yet no firm evidence identifying these factors exists.

Recent studies demonstrating inhibition of lymphocyte mitogenesis by the polyamines spermine and spermidine, which are present in human seminal plasma at high concentrations (56–1418 μg/ml and 15–20 μg/ml respectively), suggests that their inhibitory activity is dependent on the presence of factors present in bovine sera (Byrd, Jacobs & Amoss, 1977; Williamson, 1984). Since the majority of studies demonstrating SP suppression have been carried out using ruminant sera in the assay systems, and since its presence may have influenced the immune responses, we undertook to examine the functional activity of human natural killer cells and lymphocyte mitogenic responses under varying culture conditions. These studies clearly show that the addition of bovine serum to the assay greatly contributes to the phenomenon of immunosuppression mediated by SP.

MATERIALS AND METHODS

Peripheral blood. Blood was collected in heparin (10 units per ml) from healthy donors, and peripheral blood mononuclear cells (PBMC) separated on lymphocyte separation medium (Lymphoprep. Nyegaard & Co.) as described previously (Böyum, 1968). The recovered PBMC were washed three times in RPMI supplemented with antibiotics (no serum), resuspended in an appropriate medium and used in assays.

Target cells. The K562 leukaemic cell line (Lozzio & Lozzio, 1975) was used as a target cell and grown as a suspension culture in RPMI media supplemented with 10% heat-inactivated newborn calf serum (RPMI-NBCS). This line was screened for mycoplasma by ourselves and Dr R. H. Leach (Mycoplasma Reference Laboratory, Norwich, UK) and shown to be free from contamination.

Seminal plasma. Aspermic seminal plasma (SP) was obtained through a haematology laboratory routinely examining samples given by males patients following vasectomy. These specimens were not collected in anticoagulant. All samples were aliquoted and stored at −80°C until assay. Freeze-thawing did not alter the immunosuppressive potency of the SP samples.

Chromium-51 release test. K562 target cells in 0·1 ml RPMI-NBCS were radio-labelled by addition of 100 μCi of sodium chromate (0·1 ml volume) (Radiochemical Centre, Amersham, Buckinghamshire, UK) for 1 h at 37°C in a 5% CO_2-95% air humidified atmosphere unless otherwise stated. The cells were then washed (three times) in RPMI-NBCS, resuspended in 10 ml of medium and incubated for a further hour at 37°C. The cells were subsequently washed (three times) in serum-free RPMI medium, counted and resuspended in one of the following media; RPMI-NBCS, RPMI + 10% fetal calf serum (RPMI-FCS), RPMI + 10% autologous human plasma (RPMI-HuPl) or HB103 serum free medium (NEN Research products), at a concentration of $1{\cdot}0 \times 10^5$ cells/ml. Assays to detect natural cytotoxicity were performed in triplicate in round bottomed microtest wells (Falcon Microtest III flexible assays plates, Becton Dickinson). K562 cells (0·1 ml per well) were incubated together with effector cells (0·1 ml per well) at the ratios indicated, and test plates were incubated at 37°C in a 5% CO_2-95% air humidified atmosphere for 4 h, and ^{51}Cr-release and cytotoxicity calculated as previously described (Rees *et al.*, 1986).

Treatment of effector cells. PBMC were collected, as described above, and resuspended at a concentration of $5{\cdot}0 \times 10^6$ cells/ml in one of the following media; RPMI-NBCS, RPMI-FCS, RPMI-HuPl or HB103. NBCS and FCS were heat-inactivated at 56°C for 30 min. The cells were incubated at 37°C in a humidified 5% CO_2-95% air atmosphere for 18 h in flat-bottomed 24-well plates (Limbro, tissue culture grade, Flow Laboratories). SP (0·1 ml) diluted 1/5 in phosphate buffered saline (PBS) was subsequently added to the cultures to give a final dilution of 1/50 (unless otherwise indicated). The cultures were exposed to SP for 1 h (unless otherwise indicated) and then harvested from the plates by gentle pipetting, washed (three times) in RPMI and finally resuspended in 1·0 ml of RPMI-NBCS, RPMI-FCS, RPMI-HuPl or HB103. The PBMC thus treated were used in cytotoxicity assays.

Mitogen stimulation assay. PBMC were collected as described above and resuspended at $2{\cdot}0 \times 10^6$ ml cells/ml in RPMI-NBCS, RPMI-FCS or HB103. SP at 1/50 final dilution was added to the lymphocytes either for 1 h before mitogen stimulation or throughout the experiment. The

lymphocytes were stimulated with phytohaemagglutinin (PHA) (0·3–5·0 μg/ml; Wellcome Diagnostics) in round-bottomed sterile microtitre plates (Gallenkamp Co. Ltd, Stockton, UK) and cultures set up in quadruplicate. The cells were incubated at 37°C in a humidified 5% CO_2-95% air atmosphere for 72 h. ^{3}H-Thymidine, 0·5 μCi, was added to each well 4 h before the end of the assay and the cells were harvested using an automated cell harvester (Scatron A.S., Norway) onto filter paper. Incorporation of ^{3}H-thymidine was measured by counting the filter paper in scintillation fluid using a β-spectrophotometer.

RESULTS

Suppression of natural cell mediated cytotoxicity (NCMC) by SP in serum-containing media. PBMC were incubated with SP at final dilutions of 1/20, 1/40 and 1/50 for 1 h before assay for NCMC against K562 targets.

Complete suppression of cytotoxicity was shown when PBMC were pre-incubated in media (RPMI) containing 10% NBCS and SP, and to a lesser, but significant degree, in medium supplemented with FCS and SP (20%–36%) (Fig. 1). The ability of SP to inhibit NCMC was considerably reduced using media containing HuPl (RPMI + 10% autologous plasma), and was completely abrogated when PBMC and SP were incubated in HB103 media. PBMC were incubated with SP (1/50 final dilution) for 1 h, 4 h, or 20 h before assay for NCMC. Figure 2 shows that incubation of lymphocytes in RPMI-NBCS suppresses NCMC by approximately 50% after 1 h incubation of lymphocytes with SP in RPMI-NBCS suppresses NCMC by approximately 50% after 1 h incubation and by 100% after 4 h and 20 h. In RPMI-FCS there is a suppression of approximately 20% after 1 h, although this increased to 70% after 20 h incubation. When the experiment was performed in RPMI-HuPl or HB103 there was little or no suppression of NCMC even following a 20 h incubation period. No toxicity to lymphocytes was observed with any of the serum-SP combinations used, as determined by trypan blue exclusion.

Pre-treatment of effector lymphocytes for 1 h with SP (1/50 final dilution) in HB103 in the presence of 2·5% or greater NBCS induced suppression of NK cell activity, but FCS-containing HB103 did not induce SP-mediated suppression under the same conditions (results not given). The NBCS and FCS used in these experiments was heat-inactivated at 56°C for 30 min. However we

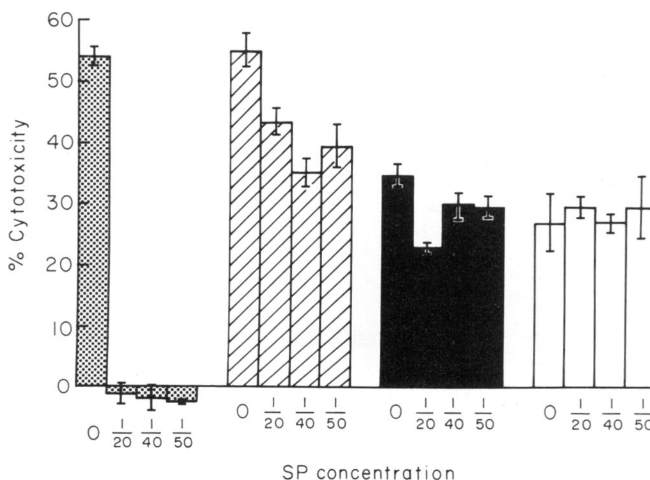

Fig. 1. The effect of culture medium on inhibition of natural cytotoxicity by seminal plasma. PBMC were treated with SP for 1 h, washed three times in RPMI before assay. The results presented are for a single experiment but are representative of those obtained in several other experiments. 10% NBCS in RPMI (▩); 10% FCS in RPMI (■); 10% Hu plasma in RPMI (■); HB103 serum-free medium (□).

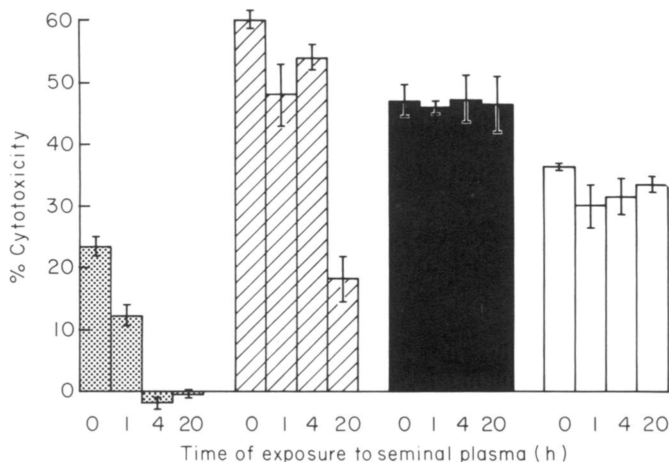

Fig. 2. The effect of exposure time to seminal plasma on suppression of natural cytotoxicity in different media. The results presented are for a single experiment but are representative of those obtained in several other experiments. 10% NBCS in RPMI (▨); 10% FCS in RPMI (▨); 10% Hu plasma in RPMI (■); HB103 serum-free medium (□).

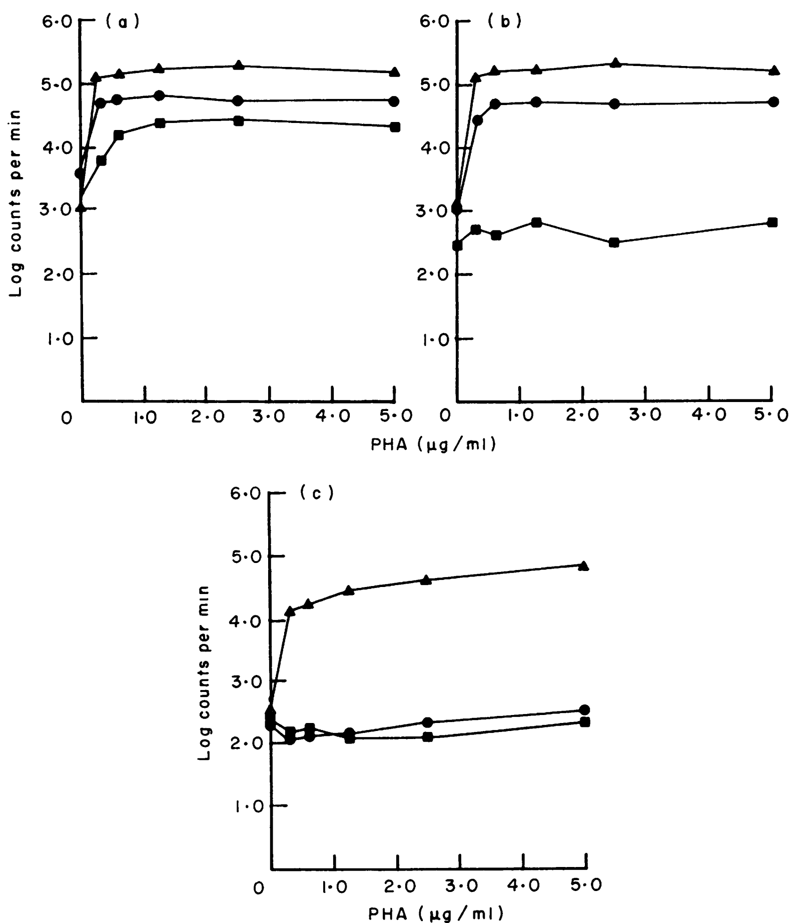

Fig. 3. Stimulation of T-lymphocyte mitogenesis with PHA in three different media: (a) no seminal plasma present; (b) PB lymphocytes pre-treated for 1 h with SP (1/50 dilution); (c) SP (1/50 dilution) present throughout assay. RPMI + 10% NBCS (■), RPMI + 10% FCS (●), HB103 medium (▲).

have unpublished data to show that a similar effect is obtained if non-heat-inactivated serum is used.

Suppression of lymphocyte mitogen response by SP in serum-containing and chemically defined media. PBMC were stimulated with PHA at concentrations ranging from 0·3–5·0 μg/ml. Assays were performed in RPMI-NBCS, RPMI-FCS and HB103, and SP (1/50 final dilution) was added to the cultures either for 1 h before stimulation or for the duration of the assay.

The results from these experiments are shown in Fig. 3 (stimulation is given on a logarithmic scale) and clearly shows the necessity of ruminant serum components for demonstrating suppression of mitogen-dependent lymphocyte activation by SP. PHA was shown to activate lymphocytes in all three media (Fig. 3a). SP inhibited activation when added to PBMC in media containing 10% NBCS for 1 h before assay, but was not inhibitory when pre-treatment was performed in RPMI plus 10% FCS or HB103 medium (Fig. 3b). However, incubation of SP with PBMC throughout the assay (72 h) inhibited completely the PBMC response to PHA in media supplemented with 10% NBCS and FCS, but only partial inhibition of mitogenesis was observed in HB103 medium (approx. 16% inhibition of control values) (Fig. 3c). These results were repeated on several occasions, with similar findings. Examination of the viability of lymphocytes after 72 h culture demonstrated >90% viability in all cultures

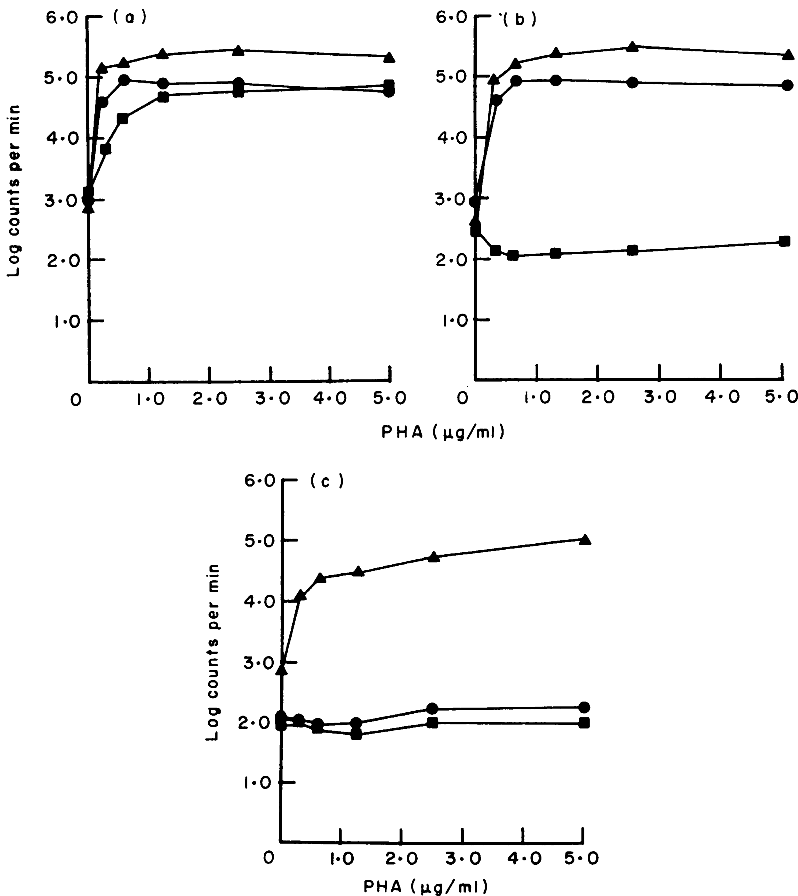

Fig. 4. Stimulation of T-lymphocyte mitogenesis with PHA in HB103 medium supplemented with NBCS or FCS. (a) No seminal plasma present; (b) Lymphocytes pre-treated for 1 h with SP (1/50 dilution) washed three times and re-cultured in the corresponding media. (c) SP (1/50 dilution) present throughout the assay. HB103+10% NBCS (■); HB103+10% FCS (●); HB103 medium (▲).

Suppression of PHA response in HB103 medium supplemented with NBCS or FCS. PBMC at a concentration of $2{\cdot}0 \times 10^6$/ml were incubated in HB103 with or without the addition of 10% NBCS or FCS, PHA and SP added at the concentrations stated above and in Fig. 4. The results, which were highly reproducible, demonstrated a distinct correlation between the suppressive influence of SP and the presence of bovine serum, and the findings were consistent with those shown using RPMI medium supplemented with NBCS or FCS (see Fig. 3). Thus, 1 h exposure of PBMC to SP in HB103 media with or without the addition of 10% FCS failed to inhibit mitogen stimulation (compare Fig. 4a and b), but pretreatment in the presence of 10% NBCS completely abolished lymphocyte responsiveness. As expected, when SP was added to cultures for the 72 h duration of the assay, complete inhibition of activation was observed in medium containing 10% NBCS or 10% FCS, but responses similar to control values were observed using serum-free HB103 medium (Fig. 4c).

DISCUSSION

The results of the present study clearly show that potent suppression of NK and T lymphocyte responses by seminal plasma (SP) components requires the addition of bovine serum factors. In serum-free culture conditions, seminal plasma was not inhibitory to lymphocyte function, but addition of exogenous serum (new born calf serum, NBCS, or fetal calf serum, FCS) resulted in significant, and often total, immunosuppression. NBCS proved more effective than FCS in inducing SP-mediated inhibition of lymphocyte function; 1 h pretreatment of effector cells in medium supplemented with NBCS and SP immediately before assaying for either NK cytotoxicity or PHA-mitogen response, was sufficient to abrogate activity. The same degree of inhibition was not obtained upon pre-treatment of effector lymphocytes in medium containing SP and 10% FCS. The mitogenic response of human PBMC in HB103 medium was not significantly reduced by the presence of SP throughout the duration of the assay.

The nature of the serum-derived and SP factors, and their mechanism of interaction remains obscure, although it is clearly shown here that the interpretation of results obtained in medium containing bovine serum is more complex than originally envisaged. Most previous studies have shown SP suppression of mitogen response using media supplemented with 10% FCS and SP (Pitout & Jordan, 1976; Byrd *et al.*, 1977; Franken & Slabber, 1981; 1982), and both reports of NK suppression used either 10% FCS (James & Szymaniec, 1985) or 10% NBCS (Rees *et al.*, 1986) as a supplement. These culture conditions may therefore have influenced the results obtained in these systems although the effect of bovine serum on other immune functions, for example, macrophage function (Chvapil *et al.*, 1977; Stankova *et al.*, 1976) have yet to be studied.

In considering the biological mechanism of SP immunosuppression, the published work on polyamine suppression of mitogen responses is relevant (Williamson, 1984). Using spermine and spermidine Byrd *et al.* (1977) demonstrated that micromolar quantities of these substances reversibly inhibited murine spleen cell mitogen activation, mixed lymphocyte responses, and the induction and suppression of cytolytic T lymphocytes. Moreover, these effects only occurred in the presence of calf or fetal calf sera. It has further been shown that polyamine inhibition of LPS-mitogenic stimulation of murine lymphocytes is abolished by the addition of spermine oxidase inhibitors (Labib & Tomasi, 1981), thus implying that polyamine oxidase activity, which is present in bovine sera, activates polyamines present in SP to molecular species possessing immunosuppressive activity. It is known (Hölttä, 1977) that prior heating of bovine serum to 60°C will affect polyamine oxidase (PAO) activity, although it is unclear whether heat-inactivation of sera (56°C, 30 min) as carried out in our experiments will affect the activity of the enzyme. It has yet to be determined whether this or similar mechanisms operate in suppressing human NK and mitogen responses *in vitro*, and whether 'activators' similar to those present in bovine sera interact with SP *in vivo* to produce inhibitory factors. PAO is not commonly found in human serum, except during pregnancy (Illei & Morgan, 1979) and this could account for the lack of suppression observed when human plasma was used in the culture medium.

The above questions need to be addressed in view of the suggestion that SP components play a role in the aetiology of acquired immune deficiency syndrome (AIDS), and indirectly in cervical

neoplasia by suppressing local immune surveillance (James & Hargreave, 1984; Rees *et al.*, 1986; Shearer & Hurtenbach, 1982; Shearer & Rabson, 1984).

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