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Involvement of interleukin 2 receptors in conceptus-derived suppression of T and B cell proliferation in horses

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Summary. The mechanism by which a horse conceptus-derived immunosuppressive factor (HCS) of $M_r > 100\,000$ inhibits lymphocyte proliferation was investigated. The factor was obtained from the culture supernatants of 20-day-old horse conceptuses; activity, identified by reduced uptake of [³H]thymidine by mitogen-stimulated lymphocytes, was greatest ($P < 0.01$) in cultures stimulated by mitogen from pokeweed. HCS also suppressed cell proliferation stimulated by phytohaemagglutinin ($P < 0.01$), but had no effect on lipopolysaccharide-stimulated cells ($P > 0.05$). Data from a fluorescence-activated cell sorter indicated that supplementation with HCS reduced the number of T cells in phytohaemagglutinin-stimulated cultures and suppressed proliferation of T and B cells in pokeweed-mitogen-stimulated cultures compared with controls. Cell proliferation was greater ($P < 0.01$) in cultures supplemented with HCS 24 h after stimulation than in those treated at the start of stimulation, and was even greater ($P < 0.01$) when cells were treated 48 h after stimulation. The removal of HCS from treated lymphocyte cultures resulted in complete recovery of cell responsiveness, and stimulated proliferation of treated cells did not differ ($P > 0.05$) from that of control cells. The addition of stimulated equine lymphocyte supernatant to cultures supplemented with HCS did not significantly increase ($P > 0.05$) cell proliferation in response to pokeweed mitogen. Addition of recombinant human interleukin 2 (rIL-2) to HCS-treated cultures did not alter the suppressive activity of HCS, although cell proliferation was greater in cultures supplemented with rIL-2 than in controls ($P < 0.01$). HCS inhibition of IL-2 receptor (IL-2R) function was investigated using an IL-2-dependent murine cytolytic T lymphocyte cell line; the fraction of HCS of $M_r > 100\,000$ had no effect ($P > 0.05$) on proliferation of IL-2-dependent murine cytolytic T lymphocyte cells induced by rIL-2. Together, these data suggest that HCS suppresses proliferation of T lymphocytes during the early stages of cell activation by inhibiting IL-2R interaction and that this suppression interferes with interactions between T cells and B cells, thereby also indirectly inhibiting proliferation of B cells. The potent immunosuppressive capacity of HCS may be one factor responsible for inhibiting cell-mediated fetal allograft rejection during pregnancy.

Keywords: interleukin 2; horse; conceptus; immunosuppression; lymphocytes; allograft

Introduction

A 30 000 kDa factor in medium conditioned by horse conceptuses has shown immunosuppressive activity (Roth *et al.*, 1990). Roth *et al.* (1991) have shown that this suppressor factor, horse

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conceptus-derived immunosuppressive factor (HCS) has $M_r > 100\,000$. High M_r components of cultures of conceptuses and of gravid uterine fluid of other domestic species, such as cows (Segerson & Bazer, 1989), sheep (Hansen *et al.*, 1987; Murray *et al.*, 1987) and pigs (Murray *et al.*, 1987), also suppress lymphocytes when used to supplement blastogenesis cultures *in vitro*. These suppressive factors may play a role in protecting the conceptus from maternal immunological attack during pregnancy. In horses, one such role may be to aid the temporary protection of the endometrial cups, which, although surrounded by lymphocytes, are not destroyed until day 120 of pregnancy (Allen, 1979). Most investigations have been directed towards identifying and characterizing uterine and conceptus-derived factors (Murray *et al.*, 1978; Godkin *et al.*, 1982; Hansen *et al.*, 1987; Newton *et al.*, 1988; McDowell *et al.*, 1990), but their functional significance is largely unknown.

Immunosuppressive mechanisms of components obtained from uterine fluid or conceptus tissue has been reported for humans (Pockley & Bolton, 1990; Saito *et al.*, 1990), mice (Clark *et al.*, 1985; Mayumi *et al.*, 1985; Clark *et al.*, 1986), sheep (Segerson, 1988) and, more recently, cows (Segerson & Libby, 1990). In these studies, suppressor activity was identified in lymphocyte cultures as reduced uptake of [3 H]thymidine by cells after mitogen stimulation. Some mitogens selectively stimulate B or T cells (Sharon, 1983), but in most immunosuppressive studies only phytohaemagglutinin, a potent T-cell activator, has been used to stimulate lymphocyte proliferation. Because cell-mediated immunity is of greater concern than humoral immunity in fetal allograft acceptance, T cells have been the focus of most attempts to elucidate suppressor factor mechanisms. The production of interleukin 2 (IL-2) and the expression of the high-affinity IL-2 receptor (IL-2R) are critical for proliferation of T cells in response to either phytohaemagglutinin or antigen (Depper *et al.*, 1984). Data from several experiments indicate that IL-2 production, IL-2R expression and their interaction are disrupted by conceptus- or uterine-derived suppressor factors in many species, including mice (Clark *et al.*, 1985, 1986), humans (Saito *et al.*, 1990; Menu & Chaouat, 1989), sheep (Segerson, 1988) and cows (Segerson & Gunsett, 1990; Segerson & Libby, 1990).

Although results from mitogen-stimulated lymphocyte proliferation assays have provided valuable information about the response of these cells to immunosuppressive factors *in vitro*, the responses of specific cell subsets have not been determined. Antibodies to unique cell subset antigens (CD4, CD5 and CD8) are available for labelling selective cell populations. Antibodies to equine lymphocytes bind either the entire T-cell population or a subset of T suppressor cells (Wyatt *et al.*, 1988). Specific fluorescent antibody labelling followed by fluorescence-activated cell sorter (FACS) analysis provides an accurate method for immunophenotypically distinguishing and quantifying cell subsets in a mixed cell population (Jackson & Warner, 1986).

The experiments described were designed (i) to examine the effect of HCS on B and T cells using various mitogens and FACS analysis; (ii) to determine the temporal relationship of HCS activity and lymphocyte activation and (iii) to define the proposed IL-2 associated mechanism of lymphocyte suppression more clearly.

Materials and Methods

Animals and conceptus cultures

The horse breeding regimen and conceptus collection techniques were as described by Roth *et al.* (1990). Briefly, conceptuses were collected by non-surgical uterine flushing on day 20 (ovulation on day 0) using sterile phosphate-buffered saline (PBS) supplemented with either 1% calf serum (Hyclone, Logan, UT) and 1% antibiotic/antimycotic (group 1), or only 1% antibiotic/antimycotic (group 2). Under sterile conditions, conceptuses were rinsed twice in their respective medium, after which group 1 conceptuses were rinsed once in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 15% fetal calf serum (FCS; Hyclone, Logan, UT; non-heat-treated, $<0.1\text{ ng ml}^{-1}$ endotoxin) and 1% antibiotic/antimycotic (RPMI+), and group 2 conceptuses were rinsed in RPMI-1640 with 1% antibiotic/antimycotic (RPMI). The three group 1 conceptuses were each placed in a 50 ml tissue culture flask containing 20 ml RPMI+. Cultures were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO₂ in air. The three group 2 conceptuses were each placed in a 50 ml tissue culture flask containing 15 ml RPMI and were incubated for 30 h under the same conditions described for group 1. Conceptus culture supernatants from each group were pooled (HCS 1 and

HCS 2), centrifuged (600 *g* for 20 min), filtered (0.2 μm) and stored in aliquots at -80°C . A lymphocyte proliferation assay was used to determine that there was no difference ($P > 0.05$) in suppressive activity between HCS 1 and HCS 2. Control medium (CM) consisted of RPMI or RPMI+ incubated, filtered and stored in a similar way.

For some experiments, HCS and CM samples were fractionated by centrifugation through microconcentrators with M_r 100 000 exclusion membranes (Amicon, Danvers, MA). Retentate fractions were rinsed twice and resuspended to their original volumes with RPMI-1640. Both fractions of CM and HCS were filtered (0.2 μm) and diluted to 600 $\mu\text{l ml}^{-1}$ in RPMI+.

Assays of lymphocyte proliferation

Unless otherwise stated, the horse lymphocyte proliferation assays were carried out as follows. Whole blood was obtained from mares by jugular venepuncture. Peripheral blood lymphocytes were isolated by Ficoll-gradient centrifugation according to McClure *et al.* (1978), resuspended in RPMI+, counted on a haemocytometer and diluted to a final concentration of 4×10^6 cells ml^{-1} . Cultures were prepared in quadruplicate for each peripheral blood lymphocyte population in 96-well round-bottomed tissue culture-treated plates (Corning Glass Works, Corning, NY, USA), each well receiving 1×10^5 cells (25 μl). The mitogens phytohaemagglutinin, pokeweed and lipopolysaccharide (Sigma, St Louis, MO, USA) were diluted in RPMI-1640 and added to stimulate cell proliferation. Maximum proliferation was induced by 10 μg phytohaemagglutinin ml^{-1} , 1 μg pokeweed ml^{-1} and 0.4 μg lipopolysaccharide ml^{-1} (final concentration in cultures). HCS or fractionated HCS was added in concentrations specified for each assay, and final well volume was 100 μl . Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air for 80–100 h before addition of [^3H]thymidine (1 μCi per well; 6.7 Ci mmol^{-1} , ICN, Irvine, CA, USA). After an additional 16 ± 2 h of culture, cells were harvested onto glass fibre filters using a Skatron automatic cell harvester (Flow Laboratories, Rockville, MD, USA). Radioactivity on the filters was determined by liquid scintillation counting.

Preliminary assays

The concentrations of phytohaemagglutinin required to induce suboptimal proliferative responses were determined by testing three peripheral blood lymphocytes samples with serial dilutions of phytohaemagglutinin (30–0.625 $\mu\text{g ml}^{-1}$). Maximum cell proliferation was achieved at a concentration of 10 $\mu\text{g ml}^{-1}$ while a suboptimal response (approximately 50% of maximum) was obtained at 1.25 $\mu\text{g ml}^{-1}$.

The suppressive potential of HCS in antigen-specific lymphocyte responses was investigated by adding HCS to two-way mixed lymphocyte reactions to ensure that components of HCS were not simply binding to the mitogens and thereby suppressing lymphocyte activation. The assays were carried out as described above except 3×10^5 cells from each of two unrelated mares were added to the wells in the absence of any mitogen. A total of six different two-way mixed lymphocyte reactions were carried out and the results, following the addition of 0, 10 and 20 μl per well HCS, were $81\,361 \pm 8190$, $53\,441 \pm 5392$ and $33\,594 \pm 3317$ (mean c.p.m. \pm SEM), respectively.

Mitogen comparison assay

The suppressive activity of HCS 2 on lipopolysaccharide- phytohaemagglutinin- and pokeweed-mitogen-stimulated lymphocytes was compared. HCS 2 was serially diluted in RPMI+ such that treated wells contained HCS 2 at 20–1.25% of total volume, whereas control wells received only RPMI+. Phytohaemagglutinin was added at the sub-optimal dose (1.25 $\mu\text{g ml}^{-1}$) to increase the sensitivity of the phytohaemagglutinin-treated cells to HCS suppression. The three mitogen assays were run simultaneously with peripheral blood lymphocytes from three randomly chosen mares. One week prior to lymphocyte collection, these mares had received an equine encephalomyelitis vaccination which is standard practice management in this area. Phytohaemagglutinin- and pokeweed-mitogen-stimulated cultures were pulsed with [^3H]thymidine at 80 h and harvested at 96 h; lipopolysaccharide-stimulated cultures were pulsed at 96 h and harvested at 112 h since we found lipopolysaccharide-treated cells require more time to achieve their maximum proliferative response.

Temporal effect of HCS

The stage of cell activation at which HCS interferes was determined by adding HCS 1 to lymphocyte cultures 0, 24 and 48 h after pokeweed mitogen stimulation of cells. The lymphocyte proliferation assay was prepared as previously described using lymphocytes from three randomly chosen mares. On the 96-well plate, triplicate rows were designated, time 0, time 24 and time 48. HCS 1 was serially diluted in RPMI+ to three final concentrations of 480, 240 and 120 $\mu\text{l ml}^{-1}$. Each of the time 0 rows were supplemented with a different dilution of HCS 1 (25 μl per well). After 24 h incubation, each of the three time 24 rows received 25 μl per well of the same three HCS 1 dilutions. At 48 h the three time 48 rows were similarly supplemented. At each treatment period, those wells not receiving HCS 1 received 25 μl RPMI+ such that final volume in all wells was 150 μl . Pokeweed mitogen was supplemented to all wells except the non-stimulated control at time 0. All wells were pulsed at 100 h and harvested at 116 h. This experiment was repeated with a total of six peripheral blood lymphocyte populations, and data from both experiments were pooled for statistical analysis.

Cells were treated with HCS 2 before mitogen stimulation to determine whether HCS-treated lymphocytes are permanently suppressed. Peripheral blood lymphocytes from two mares were prepared as described for the lymphocyte proliferation assays. The pre-stimulated cultures consisted of four treatments set up in sterile cryovials as follows: (i) non-stimulated – 150 μ l cells + 450 μ l RPMI+; (ii) control – 300 μ l cells + 900 μ l RPMI+; (iii) HCS 10 – 120 μ l HCS 2 + 300 μ l cells + 780 μ l RPMI+; (iv) HCS 5 – 60 μ l HCS 2 + 300 μ l cells + 840 μ l RPMI+. Vials were vortexed and incubated at 37°C with loosened caps for 24 h. At the end of incubation, cells were washed twice in RPMI+ (400 μ l for 10 min) and resuspended at 2×10^6 cells ml^{-1} . Cells from each prestimulated culture were pipetted into wells (50 μ l per well) and treated with 25 μ l RPMI+ containing 0 μ l (pretreated only cells), 5 μ l (pre/post-treated 5) or 10 μ l (pre/post-treated 10) HCS 2. Pokeweed mitogen was added to all wells except the non-stimulated controls and the assay completed as previously described.

Supplementation with equine lymphocyte supernatant

Equine peripheral blood lymphocyte supernatant was generated by combining peripheral blood lymphocytes from three mares, diluting cells in RPMI+ to a concentration of $5 \times 10^6 \text{ ml}^{-1}$ (10 ml total), adding 100 μ g phytohaemagglutinin, incubating for 6 h at 37°C and centrifuging at 400 g for 10 min. The cells were resuspended in 10 ml fresh RPMI+, incubated for 18 h and again centrifuged at 600 g for 10 min to obtain peripheral blood lymphocyte supernatant. Control medium (CM) consisted of 10 ml of the same RPMI+ incubated for 18 h. The peripheral blood lymphocyte supernatant assay was prepared with three lymphocyte populations. Two 96-well plates were set up with serial dilutions of HCS 2 (10–0.625 μ l per well). Cells were added to wells (1×10^5 per well), and pokeweed mitogen was added to all wells except the non-stimulated controls. All wells of one plate received 50 μ l peripheral blood lymphocyte supernatant, whereas all wells of the second plate received 50 μ l CM. After 24 h of culture, all wells received another 40 μ l of their respective medium, bringing the total well volume to 150 μ l and the assay was completed as previously described.

Supplementation with IL-2

Because recombinant human interleukin-2 (rIL-2) will interact with horse IL-2R (Fenwick *et al.*, 1988), rIL-2 (Cetus Corp., Emeryville, CA) was used in two assays designed to determine whether supplementation of IL-2 to HCS-treated lymphocytes would override the suppressive effect of HCS. Peripheral blood lymphocytes from three mares were used. In the first experiment, two sets of quadruplicate wells of each peripheral blood lymphocyte population were supplemented with 3, 6, 12 and 24 μ l of HCS 1. One set of each quadruplicate pair also received rIL-2 (200 U ml^{-1}). Suboptimal phytohaemagglutinin was added to all wells.

In the second experiment, HCS 2 was added to cultures at 15% of well volume and cell proliferation was stimulated with the suboptimal dose of phytohaemagglutinin. Serial dilutions of rIL-2 (50–0.78 U ml^{-1}) were also added to cultures. Non-stimulated cells were supplemented with rIL-2 as controls. The positive control consisted of cells, phytohaemagglutinin and rIL-2, but no HCS.

IL-2-dependent murine cytolytic T-lymphocyte cell line (CTLL-2 cells).

Unlike resting lymphocytes, CTLL-2 cells continuously express the high-affinity IL-2R complex and the addition of small concentrations of IL-2 will immediately stimulate these cells to proliferate, whereas a deficiency of IL-2 will lead to their rapid demise (Gillis *et al.*, 1978). The effect of HCS on IL-2R function was investigated by adding fractionated HCS 2 to CTLL-2 cell cultures. Triplicate wells in each row of a 96-well plate were prepared with four serial dilutions of rIL-2 (12.5 to 1.56 U ml^{-1}). One row served as a standard and each well received 50 μ l of RPMI+, the wells in the remaining rows received one of the two fractions of HCS 2 or CM (20% of final well volume). CTLL-2 cells were added to all wells (2000 cells per well) and cultures were incubated for 24 h before pulsing with [^3H]thymidine (1 μCi per well). Cultures were harvested 4 h after pulsing and radioactivity was determined by liquid scintillation counting.

Mouse splenocytes

Mouse splenocytes, obtained from spleen homogenates by Ficoll-gradient centrifugation, were rinsed twice with Hank's balanced salt solution (HBSS; Hyclone, Logan, UT) and resuspended in RPMI+ at 7×10^6 cells ml^{-1} . Splenocytes were added to two 96-well plates in 25 μ l and were stimulated by the addition of 50 μ l pokeweed mitogen, phytohaemagglutinin or Concanavalin A (ConA; Sigma, St Louis, MO) at 5, 2.5 and 5 $\mu\text{g ml}^{-1}$, respectively (final concentration in wells). Fractionated HCS 2 was added to treated wells of one plate (20% of well volume), whereas stimulated control wells received the same volume of each CM fraction. Cells were pulsed at 88 h and harvested at 100 h.

FACS analysis

FACS analysis was used to determine the effect of HCS 2 on proliferation of specific lymphocyte subsets. HCS-treated cell populations were compared with non-treated cells using three antibodies to different equine lymphocyte

markers. The monoclonal antibodies (mAb) HT23A and HT14A (pan-T and T-suppressor-cell subset antibodies, respectively; VMRD, Pullman, WA), and a fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-equine IgG antibody (B-cell antibody) were used. The conjugate for the mAbs was an FITC-conjugated goat anti-mouse IgG antibody (VMRD, Pullman, WA). Two equine peripheral blood lymphocyte populations were analysed. For each population, five tissue culture flasks were prepared as follows: (i) Control – 4 ml RPMI +; (ii) phytohaemagglutinin = phytohaemagglutinin + 4 ml RPMI +; (iii) pokeweed = pokeweed mitogen + 4 ml RPMI +; (iv) phytohaemagglutinin + = phytohaemagglutinin + 500 μ l HCS 2 + 3.5 ml RPMI +; (v) pokeweed + = pokeweed mitogen + 500 μ l HCS 2 + 3.5 ml RPMI +. To each flask, 1 ml of cells suspended in RPMI + at 5×10^6 cells ml⁻¹ was added; flasks were incubated for 96 h and cell concentration was determined using a haemocytometer. The cell suspension in each flask was divided into five sterile tubes (1 ml per tube): (1) autofluorescent control, (2) FITC control, (3) mAb HT23A, (4) mAb HT14A and (5) IgG-FITC. The cells were pelleted (400 g for 10 min) and rinsed with 3 ml PBS. The monoclonal antibodies, HT23A and HT14A, were diluted 1:1000 in PBS supplemented with 10% goat serum (PBS-GS), whereas the polyclonal goat anti-equine IgG-FITC and goat anti-mouse IgG-FITC antibodies were diluted 1:400 in PBS-GS. They were then added to appropriate tubes (50 μ l per tube); the autofluorescent and FITC controls received 50 μ l cold PBS-GA and the tubes were vortexed and incubated on ice for 1 h. All samples were washed with 3 ml ice cold PBS-GS and after decanting, 75 μ l cold 1% *p*-formaldehyde was added to the IgG-FITC tubes to fix cells, which were then vortexed and stored at 4°C. All remaining tubes, except the autofluorescent controls, received 50 μ l of diluted goat anti-mouse IgG-FITC; autofluorescent controls received 50 μ l PBS-GS. After vortexing, cells were incubated on ice for 1 h, then washed and fixed as described for the IgG-FITC tubes. Samples were stored at 4°C for 48 h, then diluted with 100 μ l PBS-EDTA and analysed on a FACS 440 (Becton Dickinson, Mountain View, CA). For each sample, 10 000 cells were analysed for both fluorescence and light scatter using a Consort 40 analyser (Becton Dickinson, Mountain View, CA). Gate settings were determined on the basis of autofluorescent and FITC controls to eliminate any non-specific or background fluorescence. Owing to between-flask variation in cell concentration following the 96 h culture, data were recorded as average cell number ml⁻¹ (final cell concentration in flask \times % of 10 000 labelled) such that results for a specific cell population would not be biased by a change in the size of other cell subset populations. The two-dimensional scatter analysis was used to compare blast cell populations between treatments. The percentage of blast cells was determined by comparing stimulated with non-stimulated FITC control samples. Gates were set such that the windowed area contained the larger cells present only in the stimulated samples. A window with the same gate settings was then applied to the HCS-treated, stimulated samples, and the percentage of cells (blast cells) in this area was recorded.

Statistical analysis

Average radioactivity (c.p.m.) and percentage of stimulated control = (average c.p.m. of experimental cultures/average c.p.m. of stimulated control cultures \times 100) for triplicate or quadruplicate wells of proliferation assays were determined. Statistical analysis was performed using data recorded as c.p.m. if the stimulated control values were the same for the treatments being tested. If the stimulated control values differed among treatment groups, data were expressed and analysed as percentages of stimulated control. Results, in the appropriate form for each assay, were analysed in a factorial arrangement by ANOVA using the general linear model procedures of the statistical analysis systems (SAS) computer package (Luginbuhl *et al.*, 1985). All main effect interactions were tested (i.e. treatment or time \times HCS or rIL-2 dose). Between-treatment groups and within treatment differences were determined by Tukey mean comparison tests. The effects of rIL-2 on HCS-treated cells and untreated cells were compared by carrying out regression analysis with data in each of the two rIL-2 supplementation assays. Treatment curves were compared and tested for differences in intercepts, linear functions and quadratic functions, and insignificant terms were dropped from the model.

Results

Mitogen comparison

The effect of HCS on lymphocyte cultures differed ($P < 0.01$) depending on the mitogen used to stimulate lymphocyte proliferation (Table 1). The results of a 3×6 factorial arrangement ANOVA and Tukey mean comparisons showed that, compared with stimulated controls, the percentage suppression of HCS-treated cell proliferation was greater ($P < 0.01$) in pokeweed-mitogen-stimulated cultures than in lipopolysaccharide- and phytohaemagglutinin-stimulated cultures. Phytohaemagglutinin-stimulated cells were suppressed ($P < 0.01$) by high concentrations of HCS (10 and 20 μ l per well), but lipopolysaccharide-stimulated cells were unaffected ($P > 0.05$) at all concentrations of HCS (within mitogen Tukey mean comparisons). Non-stimulated control values were significantly greater in the phytohaemagglutinin and pokeweed stimulated assays, probably owing to vaccines received by the horses 1 week before lymphocyte collection.

Table 1. Effect of horse conceptus conditioned medium 2 (HCS 2) on mitogen-stimulated lymphocytes in horses

HCS 2 (μ l per well)	Mitogen		
	Lipopolysaccharide ^a	Phytohaemagglutinin ^b	Pokeweed mitogen ^c
0 (control)	21 184 \pm 2713	76 648 \pm 18 131	77 111 \pm 19 572
1.25	20 180 \pm 3553	72 660 \pm 17 789	63 138 \pm 13 418*
2.5	19 446 \pm 788	72 762 \pm 16 364	43 365 \pm 9 260*
5	18 799 \pm 1420	68 037 \pm 14 010	30 595 \pm 5 274*
10	20 700 \pm 1117	58 726 \pm 13 552*	22 271 \pm 2 677*
20	19 187 \pm 682	48 905 \pm 11 160*	12 155 \pm 1 094*

Values are mean c.p.m. \pm SEM for three lymphocyte populations. Non-stimulated values for lipopolysaccharide, phytohaemagglutinin and pokeweed mitogen were 8990 \pm 822, 5657 \pm 578 and 14 712 \pm 843, respectively.

^{a-c}Different superscripts designate between-mitogen differences in overall suppressor activity ($P < 0.01$) as determined by Tukey mean comparisons using data expressed as % of control. There was a significant mitogen \times HCS interaction ($P < 0.01$).

*Differs from control ($P < 0.01$); within mitogen Tukey mean comparisons.

Temporal effect of HCS

Suppressive activity of HCS decreased ($P < 0.001$) if HCS 1 was added to lymphocyte cultures after the start of pokeweed mitogen stimulation (Fig. 1). Although still suppressive, HCS added to cultures 24 h after the start of stimulation was less ($P < 0.01$) effective than if added at time 0. By 48 h after cell stimulation, the addition of HCS to the cultures resulted in only a slight decrease in proliferation, much less ($P < 0.01$) than that observed when HCS was added at time 24. There were no main effect interactions ($P > 0.05$).

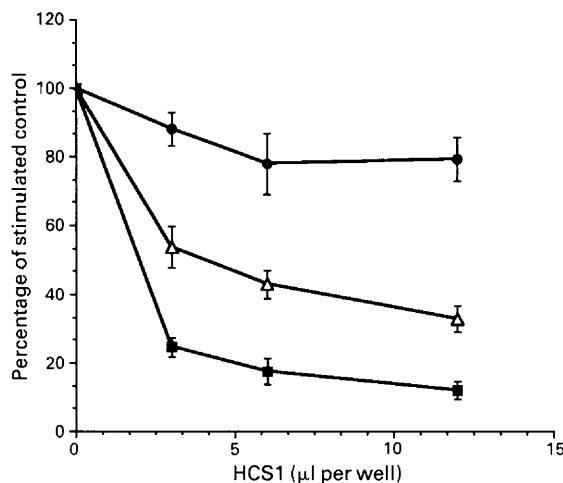


Fig. 1. Effect of supplementation with horse conceptus conditioned medium 1 (HCS 1) on pokeweed-mitogen-stimulated horse lymphocyte cultures at 0 (■), 24 (△) and 48 (●) h after the start of culture. Values represent mean % of stimulated control \pm SEM for between-lymphocyte variation ($n = 6$). Overall differences in suppression between all times evaluated were observed ($P < 0.01$; Tukey mean comparisons). There were no significant interactions ($P > 0.05$).

Lymphocytes treated with HCS 2 could respond maximally to pokeweed mitogen stimulation (Fig. 2). Proliferation of cultures pretreated with 5 or 10 μ l HCS per well did not differ ($P > 0.05$) from that of the stimulated control. However, the proliferative response of pretreated cells to which

5 and 10 μl per well HCS was again added during mitogen stimulation (before and during stimulation) was much less ($P < 0.01$) than that of both the pretreated only cells and the stimulated control.

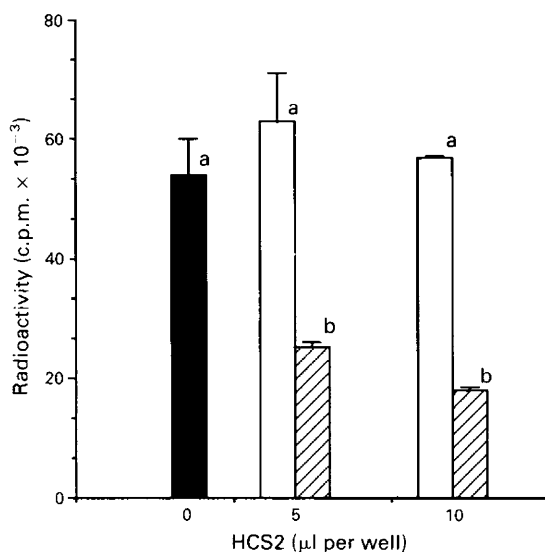


Fig. 2. Response to stimulation after incubation of horse lymphocytes with horse conceptus conditioned medium 2 (HCS 2). Values represent mean c.p.m. \pm SEM for two lymphocyte populations. Compared with the stimulated control (no HCS; ■), there was no difference ($P > 0.05$) in the proliferative responses of cells treated with HCS 2, washed and subsequently stimulated (□), while the responses of cells treated with HCS 2 before and during stimulation (▨) were suppressed ($P < 0.01$). Different superscripts denote differences in [^3H]thymidine incorporation ($P < 0.01$; Tukey mean comparisons).

Equine cell supernatant and IL-2 supplementation

Phytohaemagglutinin-stimulated peripheral blood lymphocyte supernatant was added to HCS 2-treated equine lymphocytes in an attempt to augment their proliferative response to pokeweed mitogen, but compared with proliferation in control medium-supplemented cultures, there was no difference ($P > 0.05$) in suppressive effect of HCS 2. The addition of an extremely high concentration of rIL-2 (200 U ml^{-1}) to HCS 1-treated cells did not override the suppressive activity of HCS 1. Although proliferation in cultures supplemented with rIL-2 was greater ($P < 0.01$) than that in cultures without rIL-2, a dose-dependent decrease in proliferation with the addition of HCS was observed even in cultures with 200 U ml^{-1} rIL-2 ($P < 0.01$). Furthermore, results from the regression analysis showed that both treatment curves were linear with the same slope ($P > 0.05$; ANOR; $y = 86.37 - 2.92x$; $R^2 = 0.84$) and only the intercepts were different ($P < 0.01$). Similarly, regression analysis was used to determine that the slopes of the two treatment curves, phytohaemagglutinin and phytohaemagglutinin + HCS, were linear and did not differ ($P > 0.05$) (Fig. 3). Again, only the intercepts were different ($P < 0.01$). The results of the 3×7 factorial ANOVA and Tukey mean comparisons showed that overall proliferation in HCS 2-treated cultures was less ($P < 0.01$) than that in phytohaemagglutinin control cultures, but greater ($P < 0.01$) than that in nonstimulated cultures.

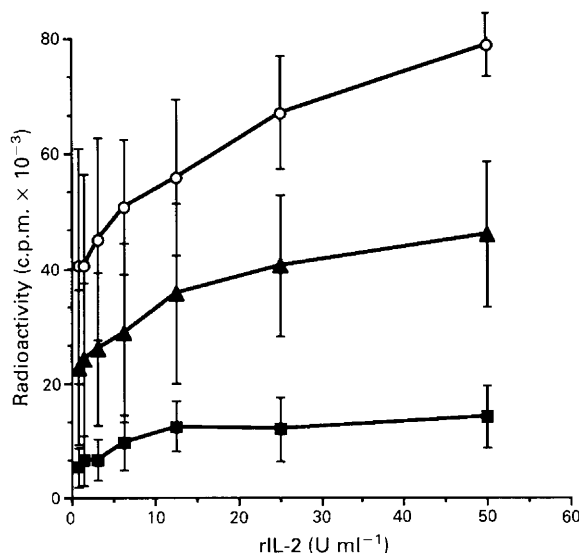


Fig. 3. Effect of recombinant human interleukin-2 (rIL-2) supplementation on non-stimulated (■) cells and on cells stimulated with phytohaemagglutinin (○), and treated with phytohaemagglutinin plus HCS 2 (▲). Data represent mean c.p.m. \pm SEM for between-lymphocyte population variation ($n = 3$). Supplementation with rIL-2 increased ($P < 0.01$) proliferation of cells within each treatment. Between-treatment differences ($P < 0.01$; Tukey mean comparisons) in overall [^3H]thymidine incorporation was noted in all treatments tested. Whereas the intercepts of all three treatment curves differ ($P < 0.01$), the linear slopes of the phytohaemagglutinin and phytohaemagglutinin plus HCS 2 curves are the same ($P > 0.05$; ANOR; $R^2 = 0.84$).

CTLL-2 cells and murine splenocytes

CTLL-2 cell proliferation in response to exogenous rIL-2 was not affected by the addition of the $M_r > 100\,000$ fraction of HCS, or the two fractions of CM (Table 2). However, the $M_r < 100\,000$ fraction of HCS did inhibit CTLL-2 proliferation ($P < 0.01$). While there was no effect of rIL-2 concentration ($P > 0.05$) except in the standard dose response, only in the standard did a decrease in rIL-2 result in a significant decrease in cell proliferation ($P < 0.05$). HCS appeared to be effective in suppressing pokeweed-mitogen-stimulated murine splenocytes as incorporation of [^3H]thymidine was less ($P < 0.01$) in cultures treated with the $M_r > 100\,000$ HCS fraction than that in cultures treated with the $M_r > 100\,000$ CM fraction. However, there were no differences in proliferation between treatments with concanavalin A or phytohaemagglutinin ($P > 0.05$).

FACS analysis

Because of the 96 h culture period before cell labelling and fixing, the cell concentration in each flask differed from the initial $1 \times 10^6 \text{ ml}^{-1}$: there was a decrease in cell number in the control flask and an increase in the mitogen-stimulated flasks. For this reason, the data are presented as final cell numbers ml^{-1} . Relative to non-stimulated controls, there was an increase in total T cells with both phytohaemagglutinin and pokeweed mitogen stimulation ($+36\,000$ and $+11\,000 \text{ cells ml}^{-1}$, respectively) (Table 3). However, a more exaggerated increase in B cells was observed with both mitogens ($+238\,000$ and $+236\,000 \text{ cells ml}^{-1}$, respectively). The addition of HCS to phytohaemagglutinin-stimulated cells resulted in a decrease of $66\,000$ T and $20\,000$ B cells ml^{-1} compared with phytohaemagglutinin-stimulated cells without HCS. When HCS was added to pokeweed mitogen-stimulated cells, a decrease in T cells also occurred ($-19\,000 \text{ cells ml}^{-1}$) and an

Table 2. Effect of fractionated horse conditioned medium 2 (HCS 2) and control medium (CM) on IL-2-dependent murine cytolytic T lymphocyte cell line (CTLL-2) and murine splenocytes

IL-2 induced CTLL-2 proliferation rIL-2 (U ml ⁻¹)		HCS 2 ^a		CM	
Standard		< 100 ^b	> 100 ^c	< 100 ^c	> 100 ^c
12.5	13.41 ± 0.45	6.28 ± 1.08	13.44 ± 1.52	10.45 ± 0.96	11.55 ± 0.17
6.25	13.48 ± 0.66	6.69 ± 0.19	13.68 ± 0.69	13.03 ± 0.51	13.89 ± 0.68
3.125	13.42 ± 0.51	6.49 ± 0.42	13.38 ± 0.77	10.34 ± 0.88	12.23 ± 0.82
1.56	10.70 ± 0.48*	5.80 ± 1.14	11.21 ± 0.98	9.05 ± 0.86	11.98 ± 0.18
Mitogen stimulated proliferation of murine splenocytes ^d					
Mitogen		< 100	> 100	< 100	> 100
Pokeweed mitogen		28.20 ± 2.03	2.79 ± 0.52**	32.67 ± 4.96	40.28 ± 1.53
Concanavalin A		52.49 ± 7.21	26.45 ± 6.76	48.32 ± 8.35	36.59 ± 9.76
Phytohaemagglutinin		13.23 ± 1.78	16.50 ± 3.14	19.44 ± 4.71	18.24 ± 4.78

Values are c.p.m. ± SEM ($\times 10^{-3}$) for triplicate wells.

^aHCS 2 and CM were separated into two fractions, M_r < 100 000 and > 100 000 as indicated by < 100 and > 100, respectively. Cultures at 20% of volume were supplemented with sample fractions.

^{b,c}Different superscripts designate between-treatment differences in [³H]thymidine incorporation ($P < 0.01$; Tukey mean comparisons). There was no treatment \times rIL-2 interaction ($P > 0.05$).

^dValues represent c.p.m. ± SEM ($\times 10^{-3}$) for quadruplicate wells.

*Designates significant difference compared with other rIL-2 concentrations within column ($P < 0.05$; Tukey).

**Designates within-row difference compared with the similar M_r fraction of CM ($P < 0.05$; Tukey).

even greater decrease in B cells was also observed ($-99\,000$ cells ml⁻¹). The presence of HCS in phytohaemagglutinin- and pokeweed mitogen-stimulated cultures decreased the T suppressor cell subset by 12 000 and 16 000 cells ml⁻¹, respectively, compared with stimulated controls. All mitogen-stimulated cell populations contained a greater number of B cells than did the control cultures, but the T-cell population decreased below control culture levels when HCS was added to the stimulated cultures ($-30\,000$ and -8000). The change in the approximate number of blast cells detected by side and forward light scatter analysis correlated with that observed with final cell concentration in the FACS cultures and with [³H]thymidine uptake data from the lymphocyte proliferation assays (Fig. 4). Compared with phytohaemagglutinin- and pokeweed mitogen-stimulated controls, the average percentage of blast cells in HCS-treated cultures decreased from 42.8 to 25.8% and from 42.2 to 8.9%, respectively.

Table 3. Fluorescence-activated cell sorter analysis of horse lymphocytes

Monoclonal antibody	Treatment ^a				
	Control	Phytohaemagglutinin	Phytohaemagglutinin +	Pokeweed	Pokeweed +
HT23A (total T)	670 ± 55	706 ± 0.5	640 ± 40	681 ± 75	662 ± 32
HT14A (T subset)	77 ± 11	162 ± 16	150 ± 28	111 ± 8	95 ± 23
IgG-FITC (B cells)	191 ± 10	429 ± 10	409 ± 17	427 ± 20	328 ± 8
Change in T cells ($\times 10^3$)		+36	-30	+11	-8
Change in B cells ($\times 10^3$)		+238	+218	+236	+137

Values represent average number of antibody-labelled cells ml⁻¹ ± SEM ($\times 10^{-3}$) for two lymphocyte populations and the average change in cell number compared with the control.

^aCulture treatments are: Control – no mitogen or HCS; phytohaemagglutinin = phytohaemagglutinin-stimulated; phytohaemagglutinin + = phytohaemagglutinin-stimulated and HCS-2-treated; pokeweed = pokeweed mitogen-stimulated; Pokeweed + = pokeweed mitogen-stimulated and HCS-2-treated.

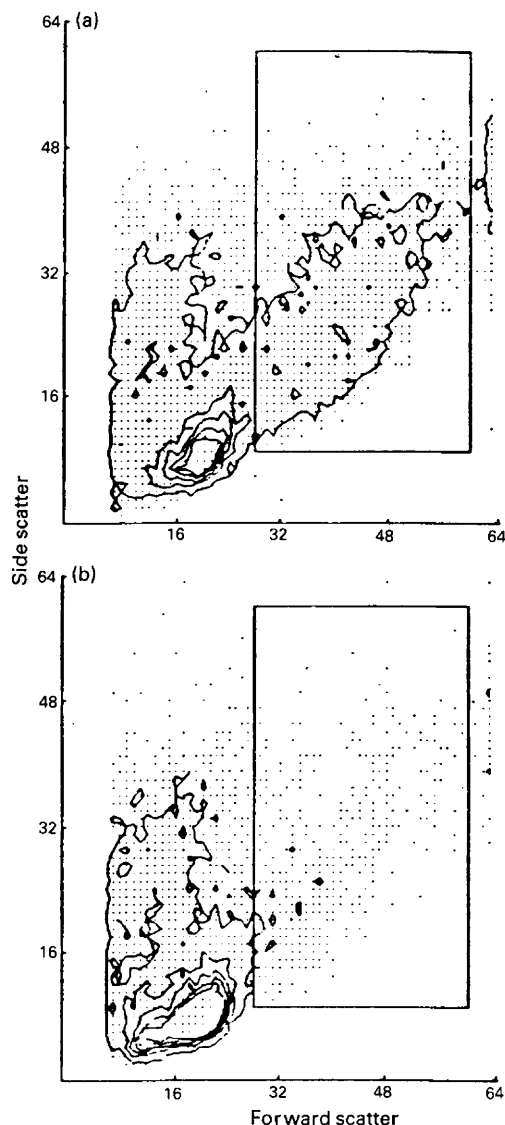


Fig. 4. Two-dimensional scatter analysis with side and forward light-scatter parameters was used to compare blast cell populations in (a) untreated and (b) HCS-treated horse lymphocyte cultures stimulated with pokeweed nitrogen. Gate settings were adjusted by comparing the mitogen-stimulated control with unstimulated control, such that the window contained the blast cells present only in the stimulated cell population. The same settings were then applied to the two-dimensional scatter plot of the HCS-treated cell population. Window in (a) shows the larger cells (blast cells, 33.3%) that were absent from the unstimulated cell population and in (b) the percentage of blast cells (5.2%).

Discussion

T cells are largely responsible for the actions of cell-mediated immunity and allograft rejection. The early developing fetus and the protective placental tissue surrounding it express antigens that are

foreign to the mother (Chatterjee-Hasrouni & Lala, 1979; Jenkinson & Owen, 1980; Billington & Burrows, 1986; Zuckerman & Head, 1986; Allen *et al.*, 1987). During successful pregnancies, fetal antigens do not initiate a cell-mediated rejection response, even though immune cells are present at the fetomaternal interface (Beer *et al.*, 1975). An immunosuppressive substance (HCS) produced by the horse conceptus and acting on T cells may inhibit the T-cell response to fetal antigens, thereby interfering with the otherwise eminent allograft rejection response. Maternal systemic immune responses would be unaffected by the localized activity of HCS, which may explain why the immune function of pregnant females is not compromised and anti-fetal antibodies are found in maternal circulation of 90% of normal equine pregnancies (Allen *et al.*, 1987), yet the conceptus remains unharmed.

Through the use of phytohaemagglutinin, a potent T-cell stimulator, pokeweed mitogen, a T-cell-dependent B-cell stimulator and lipopolysaccharide, a T-cell-independent B-cell stimulator (Sharon, 1983; Jelinek & Lipsky, 1987), we have obtained evidence indicating that the T-cell population of horses is directly affected by HCS. Possibly owing to the potent stimulatory effect of phytohaemagglutinin on T cells, relatively high concentrations (10–20%) of HCS were required before a decrease in cell proliferation was observed. Supporting evidence for the suppression of T cells by HCS was provided by FACS analysis which showed that the addition of HCS to phytohaemagglutinin-stimulated cultures greatly decreased the number of T cells, yet only slightly affected B cells. In contrast to phytohaemagglutinin-stimulated cultures, pokeweed mitogen-stimulated cells appeared to be highly vulnerable to HCS suppression as indicated by a rapid decline in their proliferative response at very low HCS concentrations. The FACS data indicated that in HCS-treated, pokeweed mitogen-stimulated cultures there was a dramatic decrease in the number of B cells. These results may be explained by the fact that B-cell proliferation depends on T-cell-derived lymphokines (Howard & Paul, 1983) and since pokeweed mitogen is a weak T-cell stimulator, the absence of even a small portion of T cells may have resulted in deficient lymphokine production and a severe reduction of B-cell proliferation. The hypothesis that HCS does not act directly on B cells is further supported by the fact that HCS is ineffective at suppressing proliferation of cultures stimulated with lipopolysaccharide. As lipopolysaccharide acts on B cells without a requirement for T cells, the effect of HCS on the T-cell population does not alter the B-cell response to lipopolysaccharide. The overall effect of HCS on mitogen-stimulated blastogenesis is most visually obvious in the FACS two-dimensional scatter analysis. Since light scatter parameters allow the sorting of cells by size and density, the blast cells can be identified. The two-dimensional scatter plot effectively demonstrates the decrease in blast cells when HCS is present in the culture.

Data from experiments relating to the temporal effect of HCS on stimulated lymphocytes support the hypothesis that HCS inhibits lymphokine production, or lymphokine receptor function. HCS is largely ineffective at suppressing cell proliferation if added 48 h after the start of stimulation. Since most cells have received both primary (mitogen) and secondary (lymphokine) stimulation by 48 h (Altman, 1990), these results indicate that HCS interferes with the initial steps of cell activation and does not affect the later events involved in proliferation. Postponing HCS treatment for 24 h appeared to allow the cells enough time to become activated by mitogen and to begin producing lymphokines (Weiss *et al.*, 1987). However, few of the cells had been fully stimulated by the necessary lymphokine–receptor interaction and thus, HCS was effective. In addition, the fact that HCS could suppress cells both 24 h after stimulation and in mixed lymphocyte reactions (see preliminary assays) clearly indicates that suppressor activity is not simply due to the binding of lectin by HCS components.

Although little is known about the effect of HCS on cells, we have shown that pretreatment of cells for 24 h with HCS, followed by thorough rinsing, resulted in a cell population with full proliferative capacity when subsequently stimulated with pokeweed mitogen. These data demonstrate that HCS is not cytotoxic and exhibits only a transient effect on lymphocyte proliferation. In addition, the fact that HCS treatment does not show any long-term effect on responsiveness of cells indicates that HCS suppression is not mediated through T suppressor cell recruitment or

activation. The FACS data support this conclusion by demonstrating a decreased number of T suppressor cells in cultures treated with HCS.

Since the administration of antibodies to IL-2 *in vivo* can extend allograft acceptance (Kirkman *et al.*, 1985) and a high IL-2 can terminate pregnancy in mice (Lala *et al.*, 1990), IL-2 may play an integral role in allograft acceptance. Results from many studies have indicated that a relationship exists between conceptus or uterine derived suppressor factors and IL-2 production (Segerson, 1988; Saito *et al.*, 1990; Segerson & Gunsett, 1990; Segerson & Libby, 1990). We tested the hypothesis that the suppressive mechanism of HCS is mediated through the inhibition of IL-2 production. Our initial attempt to overcome a potential deficiency of IL-2 in HCS-treated cultures, by supplementing the cultures with peripheral blood lymphocyte supernatant, was not particularly effective. Since it has been shown that stimulated equine lymphocyte supernatant contains the lymphokines required to stimulate IL-2-dependent cell responses (Magnuson *et al.*, 1984), we expected our peripheral blood lymphocyte supernatant to contain high levels of IL-2, but efforts to detect it were unsuccessful because the murine-derived CTLL-2 cells did not respond to equine peripheral blood lymphocyte supernatant.

Our attempt to override the suppressive activity of HCS by supplementing cultures with rIL-2 also proved unsuccessful. The fact that HCS suppressed proliferation of cells, even in the presence of an overabundance of rIL-2, demonstrates that HCS suppression is not solely a result of deficient IL-2 production. Furthermore, because the slopes of the treatment curves do not differ, we can conclude that the suppressive activity of HCS remains the same, regardless of the high IL-2 concentration in the culture. Similarly, the relative difference between the phytohaemagglutinin and phytohaemagglutinin + HCS curves (Fig. 3) remained constant at all levels of rIL-2 indicating that, while the effect of rIL-2 was similar in both treatments, there were fewer cells that could respond to the rIL-2 in the phytohaemagglutinin + HCS cultures. Because a suboptimal dose of phytohaemagglutinin was used in the cultures, there was an IL-2 deficiency and the enhanced proliferation observed in the rIL-2-supplemented cultures simply reflected the effect of sufficient IL-2 on those cells not suppressed by HCS. One explanation for the inability of rIL-2 to overcome HCS-induced suppression is that HCS inhibits IL-2R activation; alternatively, HCS may be interfering with the necessary interactions between IL-2 and IL-2R.

The latter hypothesis was tested using CTLL-2 cells that continuously express the high-affinity IL-2R. While the $M_r < 100\,000$ fraction of HCS inhibited CTLL-2 cell proliferation, possibly owing to hormones or interferons produced by the horse conceptus or both, the $M_r > 100\,000$ fraction containing HCS exerted no suppressive effect on the CTLL-2 cell response to rIL-2. The fact that murine splenocytes are suppressed if treated with the same concentration of the $M_r > 100\,000$ fraction of HCS indicates that the ineffectiveness of HCS on CTLL-2 cells is not simply due to species specificity of the suppressor factor. These results suggest that HCS interferes with cellular events before the expression of the high-affinity IL-2R, but cannot suppress cell proliferation after IL-2R expression. However, it is possible that the CTLL-2 cells differ significantly from T cells found *in vivo*, and thus may not be vulnerable to the suppressor activity of HCS.

Together, the results from rIL-2 supplementation (Fig. 3 and Table 2) suggest that, in cultures treated with HCS, there are fewer IL-2R-expressing cells that can respond to rIL-2 than in controls. These results are in agreement with reports by Bulmer & Johnson (1986) who found that T cells in human decidua do not express IL-2Rs. It is not known whether IL-2R expression is inhibited by HCS directly or indirectly through the interference of HCS with other early events in T-cell activation. For example, suppression may result from an alteration in the production or function of IL-1. Such a mechanism of suppression has been suggested for a human placental protein (Pockley & Bolton, 1990).

We reported the production of an immunosuppressive factor produced by day 10 to day 26 horse conceptuses (Roth *et al.*, 1990). In that study, the conceptus cultures were performed as described here for obtaining HCS 1. In this study, we cultured day 20 conceptuses in medium without FCS, thereby obtaining HCS 2. The immunosuppressive characteristics of HCS 1 and 2

appear to be similar, indicating that supplementation of FCS to the culture medium is not required by horse conceptus tissue for the production of the suppressor factor. HCS has an $M_r > 100\,000$, suppresses proliferation of T cells and seems to suppress B lymphocytes indirectly. This non-cytotoxic suppressor factor is effective only if present during the early stages of lymphocyte activation and its effect on cells is reversible. HCS suppression is highly dependent on its presence during the early stages of cell activation and could explain why the suppressive factors are produced as early as day 10 of pregnancy (Roth *et al.*, 1990). Our results suggest that the factor(s) suppress cell proliferation by preventing IL-2R activation. In view of the fact that T cells are largely responsible for allograft rejection, and the interaction between IL-2 and IL-2R is a prerequisite for complete T-cell activation, we hypothesize that this suppressor factor disrupts the T-cell-mediated allograft rejection that might otherwise terminate pregnancy by inhibiting IL-2R function.

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