Regulation of leukocyte interleukin 2 and interleukin 2 receptor gene expression by rabbit blastocoelic fluid

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The mechanisms underlying the inhibition of lymphocyte proliferative response by rabbit blastocoelic fluid collected on day 12 of embryonic development were investigated. Treatment with blastocoelic fluid, even in the presence of concanavalin A, maintains lymphocytes in a quiescent state by preventing cell entry into the S phase of the cell cycle. Gene expression of interleukin 2 receptor is completely blocked by treatment with blastocoelic fluid as are the secretion and gene expression of interleukin 2. Addition of interleukin 2 to prestimulated interleukin 2 receptor positive lymphocytes failed to downregulate the expression of high-affinity interleukin 2 receptor and completely abolished the embryonic fluid-mediated inhibitory effect on [³H]thymidine incorporation. Taken together, these results suggest that embryonic fluid has differential inhibitory effects, depending on the activation state of the lymphocytes. Nevertheless, inhibition of interleukin 2 and interleukin 2 receptor expression by embryonic fluid restrains immune cell activity and therefore can be implicated in the survival of the fetal semi-allograft.

Introduction

Implantation of embryos in certain mammals, such as humans and rabbits, involves cell fusion between the embryo and the uterine endometrial cells, which favours embryo–uterine exchanges (Schlafke and Enders, 1975; Morin *et al.*, 1992). A significant accumulation of leukocytes in the newly formed decidua has been demonstrated (Clark *et al.*, 1984) in some animals (including rats, mice and humans; see Stewart, 1991, for a review), leading to close contact between the embryonic trophoblast and the maternal immune system. Thus, despite the fact that trophoblastic MHC class I antigens are apparently not in direct contact with maternal blood (Redman *et al.*, 1984; Hunt *et al.*, 1988), embryos are exposed to maternal immune cells in the decidua, such as natural killer (NK)-like cells (Croy *et al.*, 1985; Gambel *et al.*, 1985), which have been associated with spontaneous abortions in mice (de Fougères and Baines, 1987; Gendron and Baines, 1988; Zheng *et al.*, 1993). Even if they are naturally resistant to NK cells *in vitro*, human and mouse trophoblastic cells have been shown to be killed by lymphocyte-activated killer (LAK) cells (Drake and Head, 1989; King and Loke, 1990). Because the uterus is, therefore, a potentially hostile environment for the embryo, immunological rejection could be expected.

It is our hypothesis that, to avoid such a disastrous fate, the embryo must locally regulate the maternal immune system. This could be accomplished in several ways: local accumulation of suppressor T cells (Clark *et al.*, 1984), an absence of major histocompatibility complex (MHC) class II antigens (Redman

et al., 1984; Ellis *et al.*, 1986), the presence of truncated MHC class I antigen on trophoblast cells (Ellis *et al.*, 1990), and local production of immunoregulatory factors, such as the ovine trophoblast protein-1 (OTP-1) (Newton *et al.*, 1989; Niwano *et al.*, 1989; Fillion *et al.*, 1991). A local rather than a systemic immunosuppression has been demonstrated (Chaouat and Monnot, 1984; Pandian *et al.*, 1988; Lambert *et al.*, 1989a). Suppressive activity affects the functional activities of NK cells (Kolb *et al.*, 1984), cytotoxic cells (Chaouat and Kolb, 1985) and macrophages (Lu *et al.*, 1984). The inhibition of the proliferative response to lectins, such as phytohaemagglutinin (PHA) and concanavalin A, by soluble immunosuppressive factors obtained from embryo culture supernatant has also been documented (Kolb *et al.*, 1984; Chaouat and Kolb, 1985; Daya and Clark, 1986a,b; Watson, 1990).

Although action of immunosuppressants specific for gestation has been described in detail, their mechanisms of action remain elusive. Blastocoelic fluid is known to have an immunosuppressive activity (Pandian *et al.*, 1988) that is modulated during rabbit embryonic development with peak activity on days 12–13 (Lambert *et al.*, 1989a). We demonstrate that this fluid, taken at day 12 of rabbit embryonic development, exerts an antiproliferative effect on lymphocytes by inhibiting interleukin 2 (IL-2) and interleukin 2 receptor (IL-2R) expression at the mRNA and protein levels.

Materials and Methods

Animals

Mature New Zealand white rabbits weighing 3.0–3.5 kg were housed separately and given free access to food and

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water. The does in oestrus were identified by the presence of oedematous and purple vulvae and chosen for the experiments. The day of oestrus was counted as day 0. On that day, the does were mated and then injected i.v. with 75 iu human chorionic gonadotrophin (hCG, APL; Ayerst Laboratories, Montréal) to ensure ovulation.

Collection of rabbit blastocoelic fluid

The procedure for collecting blastocoelic fluid is as described by Lambert *et al.* (1989b). Briefly, at day 12 of pregnancy, the rabbits were killed by an overdose of Euthanyl (MTC Pharmaceutique Ltd, Cambridge, Ontario) and the uteri were removed. Blastocoelic fluid from rabbits at day 12 of pregnancy was then aspirated by a syringe from the cavities of the embryos and centrifuged (1650 *g* for 10 min). Supernatants from day-12 blastocoelic fluid from the same female were pooled and kept frozen at -20°C until use.

Preparation of lymphocytes

Rabbit blastocoelic fluid taken at day 12 of embryonic development is known to prevent [^3H]thymidine uptake by heterologous (human) or autologous lymphocytes (Pandian *et al.*, 1988; Lambert *et al.*, 1989a). Because the effect of day-12 blastocoelic fluid was the same in heterologous conditions (rabbit day-12 blastocoelic fluid versus human lymphocytes) as in autologous conditions (day-12 blastocoelic fluid and lymphocytes from the same rabbit) and because human blood is easier to obtain, all experiments described here were conducted on human lymphocytes.

Blood from healthy volunteers was collected in sterile tubes containing heparin and diluted two-fold with sterile Hank's balanced salt solution (HBSS) in 50 ml conical tubes (Falcon Beckton-Dickinson, Lincoln Park, NJ). Peripheral blood lymphocytes were prepared using Histopaque 1077 (Sigma, Mississauga, Ontario) density gradient. Histopaque (10 ml) was added to the bottom of the tube with a sterile Pasteur pipette. The tube was then centrifuged at 800 *g* for 20 min at room temperature. The peripheral blood lymphocytes collected at the interface were washed twice with HBSS and were then resuspended (2×10^6 cells ml^{-1}) in RPMI 1640 containing glutamine, streptomycin (100 mg ml^{-1}), and penicillin (100 iu ml^{-1}) (all from Flow Laboratories, Montréal), and 10% fetal bovine serum (FBS).

Cell cultures

The cells (2×10^5 in 100 μl of cell suspension) were placed in a 96-well flat-bottomed microtitre plate (Falcon) with 25 μl of concanavalin A (5 μg ml^{-1} final concentration), 50 μl day-12 blastocoelic fluid or RPMI 1640 (negative control) and 50 μl RPMI 1640 containing FBS. The final concentration of FBS was 6.7% (v/v). The plates were incubated at 37°C under a humidified atmosphere and 5% CO_2 for 48 h. Ten microlitres of 0.02 mCi [^3H]thymidine ml^{-1} (2.0 Ci mmol^{-1} ; New England Nuclear, Lachine, Québec) was then added, and the incubation was continued for a further 18–24 h. The cells were harvested on glass fibre filters (Wallac, Turku, Finland) with a

multiple cell harvester (Skatron, Lier, Norway), and the filter discs corresponding to each well were placed into a scintillation vial with 3 ml Formula 963 (aqueous scintillation cocktail; Dupont, New England Nuclear, Lachine, Quebec). The incorporated radioactivity was then evaluated by liquid scintillation counter and calculated as described by Pandian *et al.* (1988) and Lambert *et al.* (1989a). For northern blot analysis and IL-2 assays, the cells were cultured in 24-well culture plates (900 μl per well; 400 μl cell suspension, 100 μl concanavalin A, 200 μl day-12 blastocoelic fluid or RPMI, and 200 μl RPMI containing FBS to a final FBS concentration of 6.7%) and treated as described above.

The cells used in the prestimulation studies were cultured in 24-well flat-bottomed culture plates (8×10^5 cells in a total volume of 900 μl) with 5 μg concanavalin A ml^{-1} . After incubating (37°C , 5% CO_2) for 72 h, the cells were harvested, washed twice in RPMI, and subcultured in 96-well flat-bottomed culture plates as described above, either with or without concanavalin A and day-12 blastocoelic fluid or RPMI. [^3H]thymidine (10 μl of a 0.02 mCi ml^{-1} solution) was added 18 h before counting by liquid scintillation.

Flow cytometry studies

Analysis of DNA content. Treated lymphocytes from 96-well culture plates were transferred into 1.5 ml polypropylene tubes and centrifuged at 600 *g* for 5 min, washed with PBS (pH 7.2) and then fixed in PBS–70% (v/v) ethanol. Fixed cells were kept at -20°C . Before analysis, the cells were centrifuged (600 *g* for 2 min), washed with PBS, and incubated for 30 min at 37°C in PBS containing RNase (40 U ml^{-1} per 10^6 cells) (Boehringer–Mannheim, Mannheim). The cells were centrifuged (600 *g* for 5 min) and resuspended in 500 μl PBS containing the DNA dye, propidium iodide (50 μg ml^{-1}) (Sigma), and incubated on ice for 30 min. Analysis of DNA content was performed using a Coulter Epics 753 pulse cytometer (Coulter, Hialeah, FL). The cells were excited at 488 nm (400 mW) and red fluorescence, measuring total DNA, was recorded over 590 nm. The percentages of cells in G0/G1, S and G2/M phases were calculated from the resulting DNA content histograms using the PAR1 program (Coulter). Debris and doublets were eliminated by plotting integrated red fluorescence and by setting a bitmap around the events on the diagonal (G0/G1, S and G2M singlets) (Dressler and Bartow, 1989). Chicken red blood cells (2.5 pg DNA per diploid nucleus), trout red blood cells (5.5 pg DNA per diploid nucleus) and human white blood cells (7.0 pg DNA per diploid nucleus) were used as reference standards (Tiersh *et al.*, 1989).

IL-2R membrane expression studies. Lymphocytes harvested from 96-well culture plates were washed twice and resuspended in 300 μl PBS (pH 7.4) before being incubated with 5 μl fluorescein isothiocyanate (FITC)-conjugated mouse (IgG1a) antihuman IL-2R α (4 $^{\circ}\text{C}$, 15 min) antibody (Coulter) or FITC-conjugated mouse IgG1a (isotypic control). Fluorescence was analysed by flow cytometry (Coulter Epics C cell sorter system).

Northern blot analysis

The lymphocytes were recovered from the 24-well tissue culture plates, pooled, and pelleted by centrifugation (800 *g* for 15 min) in 50 ml polypropylene tubes (Falcon). Total RNA was extracted using the method of Chomczynski and Sacchi (1987) and the samples were subsequently analysed by blot hybridization using Hybond-N (Amersham, Oakville, Ontario) as transfer membrane. cDNAs encoding human IL-2 (obtained from American Tissue Culture Collection ATTC: no. 67618), human IL-2 α (generously provided by W. C. Greene, Gladstone Institute, San Francisco, CA), or human GAPDH were digested with the appropriate restriction enzymes and the purified inserts were labelled with the Quick Prime kit (Pharmacia, Baie d'Urfe, Quebec) according to the manufacturer's procedure with $\alpha^{32}\text{P}$ dCTP (3000 Ci mmol $^{-1}$, Amersham). The probes were purified as described by Chapdelaine *et al.* (1990).

IL-2 assays

Supernatants of treated or control cells (cultured in 24-well tissue culture plates) were taken at various times and kept at -20°C until use. Interleukin 2 was assayed with an ELISA kit detecting the human cytokine (Cayman Chemical Company, Ann Harbor, MI) according to the manufacturer's guidelines.

Treatment with cytokines

Human recombinant IL-1 α , IL-4, IL-6, and tumour necrosis factor α (TNF- α) were purchased from R&D systems and IL-2 (2×10^6 U mg $^{-1}$) from Boehringer-Mannheim. Prestimulated cells were washed twice in RPMI and resuspended in RPMI-FBS (2×10^6 cells ml $^{-1}$) before being subcultured with concanavalin A alone or in combination with day-12 blastocoelic fluid. Recombinant cytokines were immediately added, and the incubation was continued for 24 h. Six hours after the beginning of the subculture, the cells were labelled with [^3H]thymidine as described. All the cytokines were tested simultaneously in the same assay using the same medium and the same peripheral blood lymphocytes for every cytokine.

Statistical analysis

Statistical treatments were performed using Duncan-Kramer multiple comparison tests or paired-sample *t* test, as indicated.

Results

Antiproliferative effect of day-12 blastocoelic fluid on human peripheral blood lymphocytes

Table 1 presents the results obtained by culturing human peripheral blood lymphocytes with concanavalin A, concanavalin A plus day-12 blastocoelic fluid, or culture medium alone (nonstimulated cells) and demonstrates the strong inhibitory effect of day-12 blastocoelic fluid (95%) on the concanavalin A-dependent T-cell proliferation. This inhibition was further characterized by undertaking cell cycle studies by flow cyto-

Table 1. Immunosuppressive properties of rabbit blastocoelic fluid collected on day 12 of pregnancy

Treatment ^a	[^3H]thymidine incorporation ^b (c.p.m.)	Inhibition ^{bc} (%)
Concanavalin A (ConA)	19 989 $\pm$ 1297	—
ConA + day-12 blastocoelic fluid	1023 $\pm$ 395	95 $\pm$ 1
Nonstimulated cells	1195 $\pm$ 558	—

^aconcanavalin A and concanavalin A + day-12 blastocoelic fluid: *n* = 32; nonstimulated cells: *n* = 14.

^bMeans $\pm$ SEM.

^c% inhibition = $\frac{\text{c.p.m. ConA} - \text{c.p.m. ConA + day-12 blastocoelic fluid}}{\text{c.p.m. ConA}} \times 100$.

Fig. 1. Cell-cycle analysis of lymphocytes treated with day-12 blastocoelic fluid. Cells were evaluated for their DNA content after incubation for 72 h in 96-well culture plates. The percentage of cells in G0/G1 (■), S (▨), and G2/M (□) is shown. Each histogram represents the mean $\pm$ SEM of four experiments performed in triplicate. **P* < 0.01, significantly different from concanavalin A (ConA) values. †*P* < 0.01, significantly different from nonstimulated (NS) values as determined by Duncan-Kramer multiple comparison test.

metric analysis of DNA content in concanavalin A-stimulated cells treated with day 12 blastocoelic fluid, concanavalin A-stimulated and nonstimulated cells. More than 80% of the concanavalin A/day-12 blastocoelic fluid-treated cells remained

in the G0/G1 phase (Fig. 1), suggesting that the addition of day-12 blastocoelic fluid to the culture maintains the lymphocytes in a quiescent state, despite the continuous presence of concanavalin A throughout the 72 h treatment. A significant inhibition of cell cycle progression ($P < 0.01$) is also reflected in the S and G2/M phases, where only $16.7 \pm 0.7\%$ and $0.8 \pm 0.2\%$ of the cell population, respectively, were propidium iodide-labelled in the concanavalin A/day-12 blastocoelic fluid-treated cells in contrast to $38.9 \pm 2.5\%$ and $13.0 \pm 1.0\%$, respectively, for concanavalin A-stimulated cells. However, despite the strong inhibitory effect, concanavalin A/day-12

Fig. 3. Interleukin 2 secretion by cells treated with (○) concanavalin A; (●) concanavalin A + day-12 blastocoelic fluid; or (■) without concanavalin A. Culture supernatants collected at various times were diluted 1:50 with RPMI-1640, frozen at -20°C and assayed for secreted interleukin 2. Each point represents the mean $\pm$ SEM of three experiments performed in duplicate. A total of eight different day-12 blastocoelic fluid samples were tested on the lymphocytes of three donors. * $P < 0.05$; ** $P < 0.01$, significantly different from concanavalin A values as determined by Duncan-Kramer multiple comparison test.

blastocoelic fluid-treated cells progressed significantly in the cell cycle compared with nonstimulated cells for the G0/G1 phase ($82.4 \pm 0.8\%$ versus $90.1 \pm 0.9\%$; $P < 0.01$) and S phase ($16.7 \pm 0.7\%$ versus $8.5 \pm 1.3\%$; $P < 0.01$). There was, however, no significant difference for the G2/M phase.

Day-12 blastocoelic fluid negatively affects IL-2 and IL-2R gene expression

It is known that IL-2R α appears at the cellular surface after T-cell activation (Leonard *et al.*, 1985; Altman *et al.*, 1990) and

Fig. 2. Expression of interleukin 2 (IL-2) receptor on day-12 blastocoelic fluid-treated cells. Peripheral blood lymphocytes were incubated for 72 h with and without concanavalin A and day-12 blastocoelic fluid in 24-well tissue culture plates. The cells were recovered and membrane expression of IL-2R α was determined by flow cytometry or northern blot analysis after extraction of RNA. (a) IL-2R membrane expression was evaluated by direct fluorescence with an anti-IL-2 receptor antibody labelled with fluorescein isothiocyanate. Non-specific fluorescence was assessed by the use of an isotypic mouse immunoglobulin labelled with the same fluorochrome. The percentage of positive cells for IL-2R membrane expression is shown as percentage fluorescence. Each histogram represents the mean $\pm$ SEM of three experiments performed in duplicate. * $P < 0.001$, significantly different from concanavalin A values, as determined by Duncan-Kramer multiple comparison test. (b, c) Total mRNA ($10 \mu\text{g}$ per lane) was hybridized with ^{32}P -labelled IL-2R or GAPDH probes and exposed at -80°C for 24 h (GAPDH) or 96 h (IL-2R).

Fig. 4. mRNA expressing interleukin 2 (IL-2) in cells treated with day-12 blastocoelic fluid. Peripheral blood lymphocytes were incubated for 72 h with and without concanavalin A and day-12 blastocoelic fluid in 24-well tissue culture plates. The cells were recovered; RNA was extracted; and northern blot analysis was performed. Total mRNA (10 μ g per lane) was hybridized with 32 P-labelled interleukin 2R (a) or GAPDH (b) probes and exposed at -80°C for 24 h (GAPDH) or 96 h (interleukin 2).

that interaction between IL-2 and its receptor is necessary for the progression of activated T-cells through the cell cycle and their subsequent proliferation (Cantrell and Smith, 1984). Day-12 blastocoelic fluid treatment prevents IL-2R α membrane expression on lymphocytes (Fig. 2a), which may reflect the quiescent state of concanavalin A/day-12 blastocoelic fluid-

treated cells observed in Fig. 1. As there was no cellular IL-2R α , no cell-cycle progression, and no proliferation in concanavalin A/day-12 blastocoelic fluid-treated cells compared with concanavalin A cells, an attempt was made to determine whether IL-2R α gene expression, normally induced by concanavalin A, was maintained quiescent by day-12 blastocoelic fluid treatment. As expected, no IL-2R α mRNA could be detected in concanavalin A/day-12 blastocoelic fluid-treated cells as determined by northern blot analysis (Fig. 2b). The double hybridization is due to some alternative splicing, and the lower band represents a transcript lacking 216 bases and is not involved in the production of an active protein (Leonard *et al.*, 1984). Because the expression of IL-2R α is known to be regulated by IL-2 (Reem and Yeh, 1984; Welte *et al.*, 1984), the low IL-2R α expression was correlated with IL-2 gene expression by measuring IL-2 production of concanavalin A/day-12 blastocoelic fluid-treated lymphocytes. Production of IL-2 was significantly inhibited ($P < 0.01$) by day-12 blastocoelic fluid (Fig. 3).

The results confirmed that day-12 blastocoelic fluid prevents IL-2 gene expression (Fig. 4) and suggest a rapid inhibitory effect of day-12 blastocoelic fluid taking place before 6 h, as shown by IL-2 secretion (Fig. 3). This is also the minimal time required to detect mRNA encoding IL-2 in concanavalin A-stimulated cells (D. Bergeron, M. Audette and R. D. Lambert, unpublished).

Exogenous IL-2 and prevention of inhibition by day-12 blastocoelic fluid

The lymphocytes were prestimulated with concanavalin A alone for 72 h to determine whether the presence of IL-2 could neutralize the day-12 blastocoelic fluid-mediated inhibition. This experimental condition was based on the demonstration (Cantrell and Smith, 1984) that lymphocyte proliferation peaks when a maximum of cell surface IL-2R expression is attained, which is 72 h after lectin activation (Leonard *et al.*, 1985; Smith, 1988). This prestimulation induces a high membrane expression of IL-2R ($68 \pm 13\%$; D. Bergeron, M. Audette and R. D. Lambert, unpublished). Although it strongly inhibits [^3H]-thymidine incorporation (Fig. 5a), day-12 blastocoelic fluid did not completely abolish cell proliferation (Fig. 5b) and failed to decrease IL-2R membrane expression ($64 \pm 4\%$; D. Bergeron, M. Audette and R. D. Lambert, unpublished). Therefore, day-12 blastocoelic fluid does not exert its inhibitory activities by downregulating IL-2R expression.

The next aim was to determine whether day-12 blastocoelic fluid interfered with proliferative transduction signals emanating from IL-2R after interaction with IL-2. The inhibitory activity of day-12 blastocoelic fluid was totally prevented by the addition of 20 U human recombinant IL-2 ml^{-1} (Fig. 6), indicating that its receptor was still functional despite the presence of day-12 blastocoelic fluid. Interleukin 1 α , IL-4, IL-6 and TNF- α are also known to play a role in lymphocyte proliferation (Durum *et al.*, 1985; Paul and Ohara, 1987; Lotz *et al.*, 1988). None of these cytokines could totally prevent the inhibitory effect of day-12 blastocoelic fluid in the range of concentrations used (Fig. 7).

Fig. 5. Proliferation profile of concanavalin A day-12 blastocoelic fluid-treated prestimulated cells as a function of time. The cells were prestimulated with concanavalin A for 72 h in 24-well culture plates before they were recultured (96-well culture plates) with (●) concanavalin A; (■) concanavalin A + day-12 blastocoelic fluid; or (○) without concanavalin A. (a) The c.p.m. per 1000 cells was calculated from data shown in (b) and total [^3H]thymidine incorporation (not shown). (b) Number of cells (haemocytometer) per well. Each histogram represents the mean $\pm$ SEM of six experiments performed in triplicate. * $P < 0.05$; ** $P < 0.01$, significantly different from concanavalin A + day-12 blastocoelic fluid values.

Discussion

The strong inhibitory effect of day-12 blastocoelic fluid cannot be attributed to cytotoxicity because, as stated by Pandian *et al.* (1988), lymphocytes treated with day-12 blastocoelic fluid showed a viability higher than 90% and pretreatment of lymphocytes with day-12 blastocoelic fluid for 24 h does not alter their ability to proliferate in response to concanavalin A when the blastocoelic fluid is removed (Pandian *et al.*, 1988). In addition, in the present study it was demonstrated that preactivated (IL-2R positive) lymphocytes responded to IL-2, despite the presence of day-12 blastocoelic fluid. The results are not due to a cytotoxic combination of FBS with day-12 blastocoelic fluid, as a strong inhibition of [^3H]thymidine incorporation is still observed in a chemically defined serum-free medium (D. Bergeron, M. Audette and R. D. Lambert, unpublished).

In this study, we demonstrated that day-12 blastocoelic fluid prevented lymphocytes from undergoing cell cycling. The differences observed in the proportions of concanavalin A/day-12 blastocoelic fluid cells in G0/G1 and S phases

Fig. 6. Exogenous interleukin 2 (IL-2) can prevent day-12 blastocoelic fluid-mediated inhibition. [^3H]thymidine incorporation was measured on prestimulated lymphocytes in the presence or absence of IL-2 (control). Various concentrations of human recombinant IL-2 were added simultaneously with concanavalin A (■) or with concanavalin A + day-12 blastocoelic fluid (□) to cells prestimulated for 72 h. The incubation was continued for an additional 24 h and incorporated radioactivity was measured. The c.p.m. per 1000 cells were calculated from data shown in Fig. 5(b). Each histogram represents the mean $\pm$ SEM of three to four experiments performed in triplicate. * $P < 0.05$, significantly different from concanavalin A + day-12 blastocoelic fluid values at no cytokine addition. † $P < 0.05$, significantly different from concanavalin A values at the same cytokine concentration.

compared with nonstimulated cells are probably due to the fact that some of the cellular events that occur after concanavalin A interaction with the cell surface occur before day-12 blastocoelic fluid-mediated inhibition takes place, which suggests that cells treated with day-12 blastocoelic fluid are stopped in their transition from G0/G1 into S phase. Because IL-2 interaction with IL-2R $\alpha\beta$ (high affinity IL-2R) is necessary for such a transition to occur (Cantrell and Smith, 1984), we postulate that day-12 blastocoelic fluid hinders the proliferation of treated lymphocytes by preventing the appearance of mRNA encoding IL-2R α , and therefore its membrane expression, indirectly by inhibiting IL-2 secretion/gene expression. All the results confirm the validity of this hypothesis.

The observation that IL-2 can reverse the inhibitory effect of day-12 blastocoelic fluid when IL-2R are present on the cell surface of leukocytes also supports this interpretation. However, IL-2 present in the incubation medium after a pre-stimulation for 12 or 24 h fails to reverse the inhibitory effect of day-12 blastocoelic fluid (D. Bergeron, M. Audette and R. D. Lambert, unpublished). Given that membrane IL-2R α expression is not initiated until 15 h of lectin stimulation (Leonard *et al.*, 1985), closely parallels [^3H]thymidine uptake (Cantrell and Smith, 1984), and peaks at 72 h after lectin activation (Leonard *et al.*, 1985; Smith, 1988), we suggest that day-12 blastocoelic fluid may be acting on IL-2R α gene expression or IL-2R α membrane expression, independently

Fig. 7. Exogenous interleukin 1 α (IL-1 α) IL-4, IL-6, or tumour necrosis factor α (TNF- α) cannot prevent day-12 blastocoelic fluid-mediated inhibition. $[^3\text{H}]$ thymidine incorporation was measured on the same prestimulated lymphocytes as those used in Fig. 6 in the presence or absence of cytokine (control). Various concentrations of cytokines were added simultaneously with (■), concanavalin A or (□), concanavalin A + day-12 blastocoelic fluid. The incubation was continued for an additional 24 h and incorporated radioactivity was measured. The c.p.m. per 1000 cells was calculated from data shown in Fig. 5b. Each histogram represents the mean $\pm$ SEM of three to four experiments done in triplicate. * $P < 0.05$; ** $P < 0.01$, significantly different from concanavalin A values at the same cytokine concentration.

from IL-2 gene transcription. The fact that day-12 blastocoelic fluid does not prevent expression of the gene encoding GAPDH indicates that a day-12 blastocoelic fluid suppressive effect is not due to a nonspecific inhibition of the transcription machinery, and is another indication that this suppression is not the result of a cytotoxic effect.

Interleukin 2 can be a mediator of pregnancy failure; indeed, in early pregnancy, NK-like cells are present in relatively large quantities in the decidua (Croy *et al.*, 1985; Gambel *et al.*, 1985) and seem to be implicated in spontaneous abortions (de Fougères and Baines, 1987; Gendron and Baines, 1988). Granulated metrial gland cells, found in rodents (Croy, 1990),

and large granulated lymphocytes, found in humans (King and Loke, 1991), are present in early decidua, both of which are related morphologically, phenotypically and functionally to NK cells (Croy, 1990; King and Loke, 1990; Lin *et al.*, 1991). Although several studies establish that naive NK cells cannot kill trophoblastic cells *in vitro* (Croy *et al.*, 1985; Drake and Head, 1989; King *et al.*, 1992), King and Loke (1990) observed that IL-2 converts decidual large granulated lymphocytes into more classic NK cells and that such a treatment greatly increases the large granulated lymphocyte lytic activity, including killing normal and malignant trophoblastic cells. It is not known whether lymphokine activated killer (LAK) cell generation occurs *in vivo*, but Lala *et al.* (1990) showed that activation of mouse maternal NK cells with IL-2 or indomethacin administration, which reduces PGE₂ production and therefore promotes IL-2 synthesis, greatly increases the abortion rate of treated animals.

We propose that day-12 blastocoelic fluid serves as a potent regulator of cell proliferation/function in quiescent as well as activated immune cells *in vivo*, before the arrival of maternal suppressor cells in the implantation region (Lambert *et al.*, 1989a). In addition, because inhibition of prestimulated cells mediated by day-12 blastocoelic fluid is reversible (D. Bergeron, M. Audette and R. D. Lambert, unpublished), this effect could be considered a transient inhibitor regulating the functions and proliferation of potentially harmful activated maternal lymphocytes coming in close contact with the placenta and the fetus via the blood circulation. Although the mechanism of action of day-12 blastocoelic fluid on prestimulated lymphocytes is unknown, it could affect an IL-2-independent proliferation pathway, as no IL-2 could be detected in the supernatant of prestimulated lymphocytes even during the first 48 h after subculture where proliferation occurs (D. Bergeron, M. Audette and R. D. Lambert, unpublished). Because IL-2-independent proliferation involves activation of protein kinase C (Takeshita *et al.*, 1988), we hypothesize that inhibition by day-12 blastocoelic fluid inhibition occurs at or downstream from this kinase.

Day-12 blastocoelic fluid is not the only inhibitor of IL-2/IL-2R production. It has been demonstrated that rat amniotic fluid (Yoshimura *et al.*, 1991) and pregnancy zone protein (Saito *et al.*, 1990) inhibit IL-2 production without interfering with IL-2R membrane expression. Nicholas and Payani (1985) showed that retroplacental serum suppresses mixed lymphocyte reaction through an inhibition of IL-2 production. Yoshida *et al.* (1990) also found that this same type of serum can cause a reduction in IL-2R membrane expression on treated lymphocytes. As in the present study involving day-12 blastocoelic fluid, this inhibitory effect can be reversed by the addition of exogenous IL-2. Skibin *et al.* (1989) reported the inhibitory effects of a human factor released from the placental chorionic membrane that prevents the expression of IL-2R. In addition, hydatidiform mole extracts are known to suppress IL-2-induced proliferation of T-cells (Bennett *et al.*, 1990).

The immunosuppressive properties of gestation-specific factors have been, in some cases, associated with their ability to bind IL-2 or IL-2R. Segerson and Libby (1990) provided experimental evidence that bovine uterine luminal protein suppresses lymphocyte proliferation by such a mechanism. Our

results demonstrate that after treatment with day-12 blastocoelic fluid, IL-2 and IL-2R proteins are not expressed, thus eliminating the participation of immunosuppressive IL-2 or IL-2R binding proteins as the causal event in the inhibitory effect. This conclusion is supported by the fact that the IL-2R present at the surface of prestimulated cells can bind IL-2 and transduce proliferative signals despite the presence of day-12 blastocoelic fluid, ruling out the intervention of at least IL-2R binding proteins.

The physiological significance of the two-way day-12 blastocoelic fluid-mediated inhibition of lymphocytes could be of great relevance. Because day-12 blastocoelic fluid-mediated immunosuppression is transient, that is, modulated during the course of embryo-fetal development (Lambert *et al.*, 1989a), the active factor(s) could prevent placental transcription of the gene encoding IL-2 (and thus IL-2 secretion) before or during the migration of immunoregulatory cells to the decidua (Slapsys and Clark, 1982) which, at a certain stage in pregnancy, are thought to replace the protective role of embryo-fetal immunosuppressive factor(s) (Lambert *et al.*, 1989a). We propose that the active molecule(s) present in day-12 blastocoelic fluid, which does not act by cytotoxic means as the effect is completely reversible (Pandian *et al.*, 1988), could participate in the survival of the fetal semi-allograft by preventing transcription of genes encoding IL-2 and IL-2R. Such a mechanism could thus prevent the accidental activation of lymphocytes located in the decidua that are potentially harmful to the fetus.

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