DISCUSSION
In all mammalian species, the trophoblast is the only fetal tissue in direct and continuous contact with maternal tissues. The ability of the trophoblast to efficiently resist conditions of allograft rejection despite its antigenic status has continued to intrigue immunologists [3-8]. One possible explanation for this resistance to immune rejection could be a selective-down regulation, by the trophoblast, of cellular lytic activity [157]. A multitude of molecules are elaborated by the trophoblast and conceivably any of these factors could exercise a modulating influence on the activity of maternal immune cells during pregnancy.

Given this scenario, this study was undertaken with the objective of understanding the plausible immunosuppressive roles played by trophoblast-derived factors in pregnancy and to identify such putative candidates. However, there arise inherent problems associated with working on trophoblast cells such as the poor survival of trophoblast cells in culture and technical problems in ensuring a regular and contamination-free supply of human placentas. Thus, human choriocarcinoma cell lines have been used as convenient model systems for the analyses of placental function.

Supernatants from human choriocarcinoma cell lines (HCS) have been shown to be capable of suppressing immune responses in vitro [27,28]. However, the effects of HCS on other immune reactions have not been reported. In this study, we have extensively worked out the functional capabilities of HCS on several immune reactions. Besides extending previous experiments on in vitro suppressive effects of HCS, we reported for the first time the suppressive influence of HCS on in vivo reactions [158].

Our data show that both the JEG-3 and JAR supernatants at a final
concentration of 5% in culture, mediate 80-90% inhibition of mitogen-induced proliferation and mixed lymphocyte reactions of human and murine lymphocytes. This clearly indicates that HCS possesses profound immunosuppressive capabilities and can also act across species to suppress responses in the murine system. Extracts and supernatants from explant cultures of murine as well as human placentas have been shown to inhibit proliferative responses of lymphocytes in vitro \[134, 143, 150\]. Our data demonstrate that HCS possesses capabilities similar to those of murine and human placental supernatants. Since gestational choriocarcinoma cells arise from trophoblast cells, culture supernatants of human choriocarcinoma cell lines and placental tissue may contain similar immunological, molecular and biological identities. Our data indicate that significant suppression of mitogen-induced proliferation of lymphocytes is encountered even at high dilutions of HCS. Placental supernatants are less potent at such low concentrations \[143\]. The high potency of HCS may be attributable to the fact that choriocarcinoma cell lines produce greater amounts of the same molecules secreted by the normal human placenta owing to malignant transformation.

Interestingly enough, HCS also causes suppression of LPS-mediated proliferation of murine splenocytes. LPS is known to preferentially stimulate mature B lymphocytes \[159\]. Thus, HCS appears to inhibit in vitro proliferation of both T as well as B cell populations. However, the variables that limit the response of B cells to LPS seem to include cell heterogeneity with regard to the maturity of B cells as well as sensitivity to growth-promoting factors from other cell types \[160\]. Similarly, proliferative responses of T cells to mitogens requires multiple, precisely timed signals from accessory cells \[159\]. Therefore, it is not possible to state that the
suppressive influence of HCS on *in vitro* proliferative responses is due to its direct effect on B and T cell proliferative events *per se*. In this context, it is worth noting that the potency of HCS-mediated suppression varies when B or T cell mitogens are used to stimulate lymphocytes; dilutions of even 1:256 cause significant suppression of Con A-induced T cell proliferation, while dilutions greater than 1:64 are required for significant suppression of LPS-mediated proliferation.

Another interesting aspect of our data is that HCS inhibits antigen-specific T cell proliferation. HCS mediates dramatic suppression of antigen-induced T cell proliferation of murine lymphocytes both *in vitro* and *in vivo*. Wolf et al have reported that JEG-3 supernatant added *in vitro* blocks antigen-induced proliferation of human peripheral blood lymphocytes [27]. However, in their studies, the investigators assessed the effect of HCS only at the high concentration of 33% in the culture. Our data indicates that HCS-mediated suppression is evident even at concentrations as low as 5% in the culture medium. Furthermore, HCS when injected *in vivo* along with antigen in CFA also arrests antigen-induced proliferation of T cells. This again reflects the high potency of HCS-derived immunosuppressive factor in inhibiting T cell proliferation in the presence of strong adjuvants such as CFA.

We have clearly ruled out the possibility that the suppressive effects of HCS on proliferative responses is due to its toxic effects on cells. The viability of lymphoid cells after incubation with HCS for 24 h is unaffected as assessed by three different parameters; thymidine incorporation, trypan blue exclusion and chromium release assay. Thus, the suppressive capability of HCS cannot be explained by cytolysis. This is further emphasized by the observation that the constitutive proliferation of human and murine
lymphoma cell lines is unaffected by the presence of HCS in the culture medium. The constitutive proliferation of B cell hybridomas is also unaltered in the presence of HCS. On the other hand, human placental supernatants (HPS) mediate profound inhibition of the constitutive proliferation of both lymphoma cell lines and B cell hybridomas. Menu \textit{et al} [145] have also reported similar findings with HPS. This discrepancy between HCS and HPS could be accounted for by the fact that different cell types of both maternal and fetal origin may be present in human placental preparations. On the other hand, choriocarcinoma cell lines, unlike crude placental preparations, are a homogeneous population of trophoblast-derived cells. Thus, while the functional capabilities of HCS and HPS are similar, such as secretion of suppressor molecules sharing biological and immunological identities, it is understandable that choriocarcinoma cell lines may also lack some immunosuppressive activities exhibited by HPS.

Human placental supernatants and murine placental extracts have been shown to effectively modulate allogeneic responses in mice \textit{in vivo} [135,146]. However, the effect of HCS on proliferative responses \textit{in vivo} had not been examined. Our data for the first time illustrate the effects of HCS \textit{in vivo} on allogeneic responses in mice [158]. Both the JEG-3 and the JAR supernatants when injected \textit{in vivo} along with allogeneic cells into the foot pads of mice, cause significant inhibition of the enlargement of the popliteal lymph nodes. Suppression is evident as early as three days after immunization, and is maximal on the fifth day after immunization, when the allogeneic response reaches its maximum in control animals. Graft versus host reaction induced by the injection of parental cells into F$_1$ recipients is a useful model for measuring the vigour of a transplantation reaction as well as for studying the stimulation and inhibition of cells undergoing an
alloresponse. One of the earliest manifestations of a GVH reaction is an activation of donor cells taking place within peripheral lymphoid tissues. Thus, the popliteal lymph node assay has proven to be a very sensitive *in vivo* test permitting accurate determination of local or acute GVHR. On the other hand, systemic GVHR leading to runting syndrome is a more severe manifestation of graft versus host reactivity.

It is conceivable that suppression of GVHR and inhibition of host-versus-graft activity in the uterus during pregnancy share similar pathways. In this context, it is worth noting that the capability of murine placental extracts to block GVHR as well as the inability of maternal lymph node and spleen cells to evoke GVHR during the course of gestation have been reported [135,161]. Human placental supernatants have also been reported to inhibit local as well as systemic GVHR [146]. No such information was available for HCS. In this study, we have shown that HCS possesses similar capabilities and can suppress both local as well as systemic GVHR [162]. HCS induces profound suppression of local GVHR to parental cells in both (C57BL/6xCBA/J)F1 and (FVBxBALB/c)F1 animals. The fact that HCS effectively inhibits GVHR in both strain combinations suggests that HCS acts in an MHC-non-restricted fashion.

The high potency of HCS is substantiated by the fact that even lethal GVHR can be suppressed by HCS. However, at both the parental cell concentrations employed to induce systemic GVHR, protection from GVHR-induced mortality was about 50-60% with about 40-50% of the mice succumbing to GVHR in spite of being administered HCS. This could be due to the use of crude supernatants in lieu of the purified factor itself. Furthermore, we lack sufficient information on the *in vivo* half-life of HCS.
In experimental transplantation situations, an allograft is recognized by active immune phenomena; the host is sensitized against the allograft antigens, the graft is attacked and rejected. At the same time it is also known that transplantation tolerance may occur by (1) preventing sensitization of the host against histocompatibility antigens of the graft, and/or (2) inducting a state of tolerance to the graft in a specific manner, without causing generalized immunosuppression [163]. Immuno-suppressive drugs have been used to facilitate the induction of transplantation tolerance in adult animals [164]. Pregnancy may be viewed as an unique case of extended survival and delayed rejection of the fetal allograft. In this context, we tested the ability of HCS to prolong rejection of tail-skin allografts. Skin grafting has been a convenient tool for transplantation biologists to investigate the properties of antigens, and the cells responding to these antigens, stimulating rejection of the allograft.

Our data illustrate that at a 95% confidence level there is a delay of about 1 week in the onset of the graft-rejection process, if the animals are treated with the JEG-3 supernatant. That the delay is limited to only 1 week may be attributable to the lack of sufficient knowledge of the *in vivo* half-life of the HCS-derived factor. Furthermore, it is worth keeping in mind that skin allografts are more rapidly rejected than other tissue grafts since (1) vascularization of the grafted tissue is delayed for many hours after grafting and it makes the undernourished graft section vulnerable to attack by the immune system of the host, (2) the skin has a relatively rich lymphatic drainage system which is rapidly re-established after grafting and (3) the skin is highly antigenic primarily because of lymphocytic infiltration and the presence of Langerhans cells [163]. Thus, it is also possible that the
Discussion

Immunosuppressive capabilities of HCS are masked in some animals by other extraneous factors accelerating the rejection process.

While HCS effectively blocks proliferative responses of lymphocytes both in vitro and in vivo, humoural responses in mice remain unaffected by HCS. Mice administered antigen along with alum both in the presence and absence of HCS produce comparable antibody titres. Thus, there appears to be a distinct dichotomy in the inhibitory capabilities of HCS. Other investigators have also reported similar findings on differential effects of placental supernatants on cell-mediated and humoural immune responses [165]. Bobe et al [134] have reported two distinct placental factors with opposite activities that either positively or negatively modulate both cell-mediated and antibody responses in mice. However, little is known about the exact mechanism of action of placental factors that may lead to such a dichotomy in the functional capabilities of the supernatants. Recently, the importance of the differential production of cytokines by T helper cells in an ongoing immune response has been highlighted. It is known that while TH₁ responses aid DTH reactions, TH₂ responses to a great extent help antibody production [166]. In light of this knowledge, we speculate that the dichotomy in the effect of HCS on humoural and cellular immune responses may be explicable by its differential effects on T-helper subsets. Studying the effect of HCS on T-independent antibody responses could also clarify the issue.

One other point to be noted is that we have assayed the antibody titres by an ELISA that does not distinguish between immunoglobulin subclasses. Placental extracts have been reported to shift isotypic balance of anti-SRBC responses in mice [167]. Whether the same is true for the effect of HCS on humoural responses needs to be ascertained.
The precise mechanisms of suppression mediated by placental suppressor factors is poorly understood at present. Both active suppression and induction of anergy can be postulated as probable mechanisms of placenta-mediated suppression. Trophoblast-derived factors have been reported to recruit and induce unique populations of suppressor cells both in vitro and in vivo [168,169]. It has been suggested that suppressor cells at the implantation site act as "security guards" blocking the maternal rejection process [17]. Suppressor T cells in the uterus capable of inhibiting the generation of cytolytic effectors and regulating the istotypic spectrum of maternal anti-paternal antibodies have also been reported [71,167]. In this context, we focussed on ascertaining whether or not HCS leads to the induction of suppressor cells. In 1984, Chaouat and Chaffaux [136] have reported an easy in vivo test for screening the effects of putative suppressor cell-inducing substances. It is based on the principle that injection of suppressive material and alloantigens promote the induction of splenic suppressors of a local graft versus host reaction. By the use of this assay, we have shown that HCS also induces suppressor cells in vivo, which when transferred to mice of another H-2 strain fail to evoke an allogeneic response. However, the phenotype of the suppressor cells needs to be ascertained. Murine placental extracts have been demonstrated to induce suppressor T cells [170]. Further experiments will be required to determine the correlation between these suppressor cells and HCS-induced suppressor cells. The precise mechanism of suppressor cell induction by HCS and its functioning also needs to be ascertained. Operationally distinct populations of macrophages have been implicated in aiding the induction of T\textsubscript{s} cells in normal immune responses [171]. Several cytokines have also been reported to confer on antigen presenting cells the capacity to induce T\textsubscript{s} cells [171]. It
would be of interest to elucidate whether similar mechanisms hold true for suppressor cell induction by HCS.

Matsuzaki et al. [155] have reported that suppression mediated by JEG-3 cell line acts on T-cell signal transduction pathway and is directed at events subsequent to IL-2 stimulation. Lymphocyte activation and subsequent proliferation are a highly complex series of sequential events. Determination of the exact point of action of HCS along the pathways of lymphocyte activation and proliferation ought to throw more light on placenta-mediated suppression and its importance to fetal survival.

The major lacuna in the understanding of placenta-mediated immunosuppressive effects has been the lack of sufficient knowledge on the biochemical nature of the suppressor molecules. The principal aim of this work has been to isolate and characterize the immunosuppressive factor derived from supernatants of a human choriocarcinoma cell line, JEG-3 (HCSf).

Several gestational hormones and glycoproteins have been reported to be immunosuppressive; some of these include human placental lactogen, progesterone, alpha-fetoprotein and human chorionic gonadotropin [74,83,94]. Choriocarcinoma cell lines are known to secrete several of these gestational hormones. To rule out possibility of suppression caused by hCG and progesterone which are present in HCS, we tested the immunosuppressive effects of HCS after incubation of the supernatants with polyclonal goat anti-hCG and anti-progesterone antibodies. These antibodies failed to reverse the inhibition caused by HCS, indicating thereby that HCS-mediated suppression is not attributable to the presence of hCG and...
progesterone in the supernatant. HCS was also seen to retain its immunosuppressive activity after heat treatment. This suggests that HCS-derived factor is unlikely to be a prostaglandin since most molecules of this family are heat-labile in nature.

Having ruled out the possibility that HCS mediates suppression through hitherto known molecules, we proceeded to purify the HCS-derived immunosuppressive factor. Purification procedures demand extensive rounds of lyophilization. Therefore, prior to subjecting HCS to fractionation on HPLC and FPLC we confirmed that the inhibitory activity is retained after lyophilization. Ultrafiltration and dialysis of HCS suggested that the active moiety was a molecule greater than 10 kDa.

HCS was initially fractionated by anion-exchange chromatography. The suppressive activity of HCS was restricted to a single fraction. Purification of this fraction by gel filtration chromatography revealed the presence of a single major moiety of about 74 kDa. SDS-PAGE analysis also confirmed the presence of a major band in the range of 70 kDa. However, further purification by reverse phase chromatography under acidic conditions revealed HCSf to be a highly hydrophilic compound of low molecular weight.

Ion-exchange chromatography separates molecules on the basis of their molecular charge. The separation proceeds because ions of opposite charge are retained to different extents. The resolution is influenced by the pH of the eluent which affects selectivity, and by the ionic strength of the buffer which mainly affects retention. Gel filtration separates molecules in decreasing order of their molecular sizes. However, both gel filtration and anion-exchange columns are unstable when run at acidic conditions [172]. Thus,
resolution of smaller molecules tagged to larger ones may not be possible with these columns. In any case, these columns provide efficient first step procedures in the purification. On the other hand, reverse phase columns separate molecules based on hydrophobic interactions and are advantageous in that they are stable between a wide range of pH, i.e., 2-7 [172]. Thus, while HCSf was purified apparently tagged to a larger molecule in the range of 70 kDa on anion and gel filtration columns, cleavage of HCSf from the larger molecule has been achieved by reverse phase HPLC carried out under acidic conditions.

That HCSf initially purified was in a form tagged to BSA, is clear from the fact that antisera raised to the suppressive moiety after purification on anion-exchange and gel filtration columns shows distinctive reactivity only to BSA and not to lysozyme or hCG in an ELISA. BSA is known to be a carrier protein and hence it is understandable that HCSf is tagged to BSA in tissue culture medium containing fetal calf serum. Clark et al have also reported similar observations with the decidual suppressor factor (DSF). They purified DSF tagged to BSA on a TSK G 3000 SW gel filtration column on HPLC and reported DSF to be in the range of 80-100 kDa. Subsequently, further purification under acidic conditions revealed DSF to be in the range of 23 kDa closely related to TGF-β [125]. The TGF-β related glioblastoma tumor culture supernatant-derived suppressive factor was also initially found to be associated with BSA and reported to be in the range of 80-100 kDa [173]. Suffice it to say that co-elution of immunosuppressive molecules with carrier moieties such as BSA in tissue culture medium finds precedence in other systems [174].

One important question that arises is: what molecules act as natural carriers
for small molecular weight immunosuppressive factors in the absence of fetal calf serum-derived BSA in the tissue culture medium as well as in vivo? Alpha-feto protein, the fetal equivalent of BSA functions as a carrier protein for several molecules in vivo [97] and can be envisaged as one plausible candidate. Uromodulin, an 85-kDa glycoprotein found in the urine of pregnant women that binds cytokines such as IL-1 and TNF-α [175], could be one other putative candidate carrier protein in vivo.

Our data shows that HCSf elutes as a single peak very early on preparative and analytical reverse phase columns clearly indicating that HCSf is highly hydrophilic in nature. Furthermore, it rules out the possibility that HCSf is related to PGE2 since these molecules are hydrophobic and elute at about 14-20 minutes when run on reverse phase columns comparable in dimensions to those used in our study and under similar solvent system [176].

Spectral analysis of HCSf reveals a hump around 280 nm suggesting the presence of aromatic amino acids in the sample. Lowry estimation suggested that only 10-15% of HCSf consists of protein moieties, however, ninhydrin estimation demonstrated that a major component of HCSf is constituted by amino acids. This apparent contradiction in the results of Lowry and ninhydrin estimation of HCSf is attributable to the varying sensitivities of the two assays. Protein estimation by the Lowry method is based on the principle that (1) the phosphomolybdate of the Folin-Ciocalteau reagent is reduced by tyrosine and tryptophan present in the protein to give a coloured complex and (2) the peptide bonds to a lesser extent react with copper ions in the alkaline solution to give a coloured complex. Thus, the intensity of the Lowry’s reaction is dependant on (1) aromatic residues in the
protein as well as the number of peptide bonds. In general, while large proteins can be estimated more or less accurately by Lowry's test, peptides are less reactive in this assay [156]. Therefore, if the test sample is a peptide such as HCSf, one would tend to grossly under-estimate the amount of protein in the sample. On the other hand, ninhydrin reagent reacts with the $\alpha$-amino group of all amino acids forming a coloured complex [177] and the ninhydrin estimation procedure is therefore a highly sensitive test for peptides.

The presence of a peptide moiety in HCSf has been confirmed by amino acid analysis. The amino acid analysis data substantiates the highly hydrophilic nature of HCSf; it comprises exclusively of hydrophilic amino acids. That the peptide moiety is a necessary requisite for inhibitory activity of HCSf is apparent by the demonstration that HCSf retains only about 30% of its activity after protease digestion. However, a total loss of the inhibitory activity after protease digestion is not observed. This may be ascribable to the presence of other additional non-peptide moieties constituting HCSf, and also important for bioactivity. Based on amino acid analysis, and assuming that the least abundant amino acid is present as a single residue, we estimate HCSf to be in the range of 5-6 kDa.

Wolf et al [27] reported the JEG-3 factor to be an immunosuppressive lipoprotein with a molecular weight of 150-200 kDa. However, in these studies fractionation of the crude JEG-3 supernatant was carried out on a primitive S-200 gel filtration column. Using both FPLC and HPLC, we have succeeded in isolating and characterizing the JEG-3-derived immunosuppressive factor to be a highly hydrophilic compound of low molecular weight.
Our study shows that HCSf may contain trace amounts of sugar. While all carbohydrates can be estimated by the phenol sulphuric acid estimation procedure, glycoproteins give only mild reactivity in this assay. Thus, HCSf may be a glycopeptide. However, further experimentation will be required to accurately identify the complete biochemical nature of HCSf.

Other immunosuppressive molecules reported to play crucial roles in pregnancy include: (1) The TGF-β2-related, 23 kDa suppressor factor derived from decidual culture supernatants [125], (2) T cell-derived suppressor factor TJ6, a glycoprotein with a molecular weight of 55 kDa [67] and (3) the progesterone-induced suppressor factor (PISF) with an apparent molecular weight of 34 kDa [178]. The molecular sizes of these compounds far exceed the estimated molecular weight of HCSf. Chaouat et al (personal communication) have characterized a small molecular weight suppressor factor from human placental supernatants. While their factor appears to be hydrophobic in nature (Chaouat et al, personal communication) HCSf is highly hydrophilic. However, the possibility of the presence of more than one immunosuppressive molecule at the feto-maternal interface cannot be ruled out. HCSf may well turn out to be one of two or more immunoregulatory molecules secreted by the placenta in vivo.

Immunosuppressive molecules have also been reported from some carcinoma cell lines. Examples include those derived from virus-transformed cell lines [179,180] and solid tumors [181]. Only a few of these factors have been completely characterized. Medoff et al [182] described a 50 kDa suppressor factor derived from malignant ascites that resembles the carcinoembryonic antigen-induced suppressor factor. Suppressor factors in the high molecular weight ranges (260, 140 and 70 kDa) have been reported
from melanoma cell lines [183]. Recently, a 47 kDa suppressor factor was isolated from culture supernatants of a T cell hybridoma derived from lymphocytes of patients who had received bone marrow transplants [184]. HCSf appears to be biochemically distinct from the malignant cell line-derived suppressor factors mentioned above. Roth et al [181] have reported a suppressor factor from a liposarcoma cell line that co-purifies with BSA. However, the active moiety has not been identified. Therefore, the exact correlation between HCSf and this factor will have to await the identification of the active moiety in the latter case. In this context, it is of interest to note that supernatants from uterine cancer cell lines such as HELA do not possess immunosuppressive capabilities [28]. Therefore, the secretion of HCSf by choriocarcinoma cell lines is unlikely to be a product of malignant transformation.

In this study, we have also ascertained the potency of purified HCSf. A 50% suppression of the mitogen-induced proliferation of both human and murine lymphocytes in vitro is seen with a final concentration of 32-64 μg/ml HCSf in the culture medium. This is far less than the effective concentrations of the crude supernatant. This loss in potency of the purified factor may be attributable to the harsh treatments to which HCS is subjected during purification or to the declining half-life of HCSf after its cleavage from BSA.

In the past decade, the stimulatory effects of cytokines on placental growth and function have been highlighted [25]. Regulation of inflammatory processes by cytokines at the site of implantation appears to initiate and promote vascularization and blood flow conducive to fetal survival [67]. In such a scenario, overstimulation of the maternal immune system can cause thrombosis and fetal loss [66]. It has therefore been suggested that local
intrauterine suppressor mechanisms may be an essential concomitant of successful pregnancy that helps maintain the balance between blood flow and nourishment required for fetal growth while preventing an exacerbated exposure to the graft rejection process [67]. Trophoblast-mediated suppression is envisaged as a highly localized phenomenon, acting within a short range, capable of specifically blocking only those types of maternal immune effector mechanisms that are deleterious to fetal survival [157]. Our study has lead to the characterization of a trophoblast-produced suppressor molecule mediating such a form of suppression. Studies such as this besides providing an insight into the complexity of fetal survival could also throw light on the mode(s) of action of immunosuppressive factors and may find applications in experimental transplantation situations.