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IN VITRO INDUCTION OF HUMAN SUPPRESSOR T CELLS BY A CHORIONIC GONADOTROPIN PREPARATION

T. FUCHS [1], L. HAMMARSTRÖM [2], C.I.E. SMITH [2] and J. BRUNDIN [1]

[1] *Department of Obstetrics and Gynecology, Danderyd Hospital, 182 88, Danderyd, and*
[2] *Department of Immunology, Wallenberg Laboratory, Lilla Frescati, S-104 05, Stockholm 50, Sweden*

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Human chorionic gonadotropin (hCG) in physiological retroplacental concentration has been shown to possess the capacity of inducing human lymphocytes which are subsequently competent to depress an antibody response induced by purified protein derivative of tuberculin, phytohemagglutinin, lipopolysaccharide and pokeweed mitogen. These suppressor cells inhibited the T cell-dependent mitogen-induced activation of lymphocytes synthesizing IgM, IgG and IgA. No suppression by hCG-induced cells was observed in Epstein-Barr virus activated cell cultures, indicating a T cell origin of the target cell. It is suggested that this may represent a mechanism for the cellular basis of an hCG-induced immunosuppressive effect in pregnancy.

INTRODUCTION

Although the fetus inherits paternal histocompatibility antigens and thus constitutes an allograft to the mother no rejection process normally occurs. Many theories have been proposed to explain this successful 'transplantation' but none have so far gained wide acceptance (see Billingham, 1971). Human chorionic gonadotropin (hCG) is a glycoprotein hormone produced in large quantities during pregnancy by the trophoblastic cells of the placenta. This hormone has been claimed to suppress mitogen induced proliferation of both T (Kaye and Jones, 1971; Adcock et al., 1973; Contractor and Davies, 1973) and B lymphocytes (Beck et al., 1977; Hammarström et al., 1979). The mechanism by which this immunosuppressive effect is exerted is, however, poorly understood (see Hammarström et al., 1979). In 1977, Murgita and co-workers demonstrated the ability of α-fetoprotein (AFP), a compound produced in large amounts by the fetus in utero, to induce suppressor T cells in the mouse. This prompted us to investigate whether a similar mechanism might be responsible for the immunosuppressive effect of hCG. In a recent study, the ability of an hCG preparation to induce suppressor T cells in mice was demonstrated (Fuchs et al., 1980). In the present study we describe the capacity of hCG to induce human suppressor T cells capable of impairing the mitogen-induced differentiation of B lymphocytes.

MATERIALS AND METHODS

Experimental procedure

Human peripheral blood was obtained from adult female non-pregnant donors receiv-

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ing no medication and separated on a Ficoll–Isopaque density gradient. The cells were either cultured untreated or in the presence of hCG (Gonadex®) or 50 μg/ml of concanavalin A (Con A). Culture was performed in 50 ml tissue culture flasks (Falcon 3013, Falcon Plastics, Oxnard, Calif.) at a cell density of 5×10^6 cells/5 ml of RPMI 1640 medium supplemented with 10% heat-inactivated unabsorbed human AB-serum. After 2 days the cultures were harvested and 10^6 pre-cultured cells added to 4×10^6 fresh autologous lymphocytes (prepared by Ficoll–Isopaque separation). Survival at the end of the 48 h pre-culture period was 60, 64 and 39%, respectively, in the groups pre-cultured without addition of stimulators, with hCG or with Con A. The admixture was cultured in 50-ml tissue culture flasks in 5 ml of RPMI 1640 medium supplemented with 10% heat-inactivated unabsorbed human AB-serum or, in the EBV (Epstein-Barr virus) experiments, with 10% heat-inactivated, unabsorbed fetal calf serum with or without the addition of various polyclonal activating ligands. After 6 days the cultures were harvested and assayed for plaque-forming cell numbers using the indirect haemolytic *Staphylococcus aureus* protein A plaque assay. Cell viability was determined by trypan blue dye exclusion.

Mitogens

Concanavalin A (Con A) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Phytohemagglutinin (PHA) was purchased from Wellcome Research Laboratories Beckenham, Kent, U.K. Lipopolysaccharide (LPS) from *Escherichia coli* 055 : B5 was obtained from Professor T. Holme, Department of Bacteriology, Karolinska Institute, Stockholm, Sweden. Purified protein derivative of tuberculin (PPD) was purchased from Statens Serum-Institut, Copenhagen, Denmark. Pokeweed mitogen (PWM) was purchased from Techtum Instruments, Umeå, Sweden. Epstein-Barr virus (EBV) (B 95-8) was kindly supplied by Professor G. Klein, Department of Tumor Biology, Karolinska Institute, Sweden. Exposure of lymphocytes to EBV was carried out as described previously (Rosén et al., 1977).

Hormones

Purified hCG (Gonadex®), phenol and preservative-free, containing 6000 IU hCG and 50 mg mannitol per ampulla was generously supplied by Leo AB, Hälsingborg, Sweden. Isoelectric focusing (PAG plates, pH 3–9) after Coumassie blue staining gave rise to 5 bands focused between pH 4.5 and 6. Pure mannitol was purchased from Sigma Chemical Co., St. Louis, Mo., USA.

Coupling of protein A to erythrocytes

Staphylococcus aureus protein A (Pharmacia) was coupled to sheep red blood cells (SRBC) using $CrCl_3$ as described by Gronowicz et al. (1976). SRBC, stored in Alserver's solution, were washed three times in 0.9% NaCl. Thereafter, one part of protein A (0.5 mg/ml) was mixed with 10 parts of $CrCl_3$ (2.5×10^{-4} M) and one part of packed SRBC. All reagents were diluted in 0.9% NaCl. The mixture was incubated at 30°C for 1 h and thereafter washed once in 0.9% NaCl and twice in balanced salt solution (BSS) and kept at 4°C. Cells were usable up to 4 days after coupling.

Plaque assay

100 μl of cells suspended in BSS were directly added together with 25 μl of protein A-coupled erythrocytes (diluted 1 : 3), 25 μl of the IgG fraction of the anti-serum diluted 1 : 30 (anti-human, μ-, γ-, α-chain Ig produced in rabbit; DAKO-Immunoglobulins Ltd., Copenhagen, Denmark) and 25 μl of SRBC-absorbed guinea pig complement (diluted 1 : 4) into 700 μl 0.5% agar in BSS containing 0.45 mg/ml DEAE-dextran. Two 0.2 ml drops of the mixture were placed on a plastic 9 cm petri dish and a 24 × 32 mm glass cover-slip was immediately placed on each drop. Dishes were incubated for 4 h at 37°C, and plaques were counted using indirect light.

Assay for T and B lymphocytes

T cell separation was performed using a slight modification (Persson et al., 1978) of the method described by Julius et al. (1973). Briefly, a cell suspension was added directly on nylon wool columns consisting of 0.6 g of the wool closely packed into 10 ml syringes. The cells were eluted with BSS supplemented with 5% FCS. In order to determine the T/B cell ratio after nylon wool filtration, isolated lymphocytes were washed several times in BSS and incubated with 0.05 ml of a fluorescein-labelled polyvalent rabbit anti-human immunoglobulin serum for 30 min at room temperature. After washing in BSS the cells were kept on ice while fluorescent cells (B-cells) were enumerated in a Leitz fluorescence microscope under direct illumination (see Hammarström et al., 1975).

Statistical analysis

Results represent mean suppressive effect of the different Ig classes. Plaque-forming cell (PFC) numbers cultured in groups containing pre-cultured cells without any pre-activating substance added represent 0% suppression. Statistical analysis of suppression was performed using the matched Student's *t*-test. PFC numbers in cultures with cells pre-cultured in the absence of hCG or Con A = *a*. PFC numbers in cultures with cells pre-cultured in the presence of hCG or Con A = *b*. Variables were tested under the assumption that no significant difference existed. Comparison was made between $(a - b)/\frac{1}{2}(a + b)$ and background $(a - a)/\frac{1}{2}(a + a) = 0$.

RESULTS

hCG induces suppressor cells

In a first set of experiments, it was tested whether the hCG preparation was capable of inducing suppressor cells. Doses of 2000 and 1000 IU/ml were chosen since we have previously shown that 2000 IU/ml of hCG is immunosuppressive when added to human lymphocyte cultures (Hammarström et al., 1979) and, furthermore, these concentrations of hCG were previously found to induce suppressor cells in mice (Fuchs et al., 1980). Con A-induced suppressor cells were used as a comparison for suppressive effect.

Table 1 illustrates the results from a representative experiment carried out with 2000 IU/ml of hCG where pre-culture of human lymphocytes with Con A or hCG resulted in the formation of cells, capable of inhibiting the activation of fresh autologous lymphocytes by PPD. In this particular experiment, hCG diminished IgM, IgG and IgA responses by 48, 37 and 66%, respectively (48, 33 and 47% when background was not subtracted),

TABLE 1

Suppressive effect of human chorionic gonadotropin

Fresh autologous lymphocytes	48 h pre-incubation	Mitogen	PFC/culture			
			IgM	IgG	IgA	0 [b]
4×10^6	0	0	1 309	1 396	1 309	0
		PPD	45 396	41 904	11 349	0
5×10^6	0	0	2 037	1 600	2 182	0
		PPD	58 200	27 645	14 841	0
4×10^6	10^6	0	145	873	1 134	0
		PPD	59 160	29 000	19 206 [a]	0
4×10^6	10^6 Con A	0	873	4 074	3 492	0
		PPD	5 238	4 785	4 930	0
4×10^6	10^6 hCG	0	582	783	2 182	0
		PPD	31 320	19 140	10 150	0

Human peripheral blood lymphocytes were cultured in the presence of 50 μg Con A/ml, 2000 IU/ml hCG or devoid of pre-activating substance. After 48 h the cells were harvested and 10^6 pre-cultured cells were mixed with 4×10^6 autologous fresh lymphocytes. The admixture was cultured in the presence of 100 μg PPD/ml and harvested on day 6. Results represent PFC/culture.

[a] 0% suppression.

[b] No developing serum added in the assay.

which should be compared with a Con A-induced suppression of 93, 98 and 92% (92, 84 and 74% when background was not subtracted). When all experiments using PPD as a stimulator were pooled ($n = 9$), the mean suppression (measured as total IgM + IgG + IgA PFC numbers) induced by hCG and Con A was 48 and 84%, respectively. Thus, pre-culture of cells with hCG for 48 h and the addition of these cells to cultures with autologous fresh cells resulted in a statistically significant ($P < 0.001$) decrease in IgM, IgG and IgA PFC numbers. No selective suppression was observed when PFC numbers of different Ig classes were compared ($P > 0.05$). Pure mannitol, when added in equimolar doses to those present in the Gonadex® did not induce any suppressive effect (data not shown). Pre-culture of lymphocytes with hCG for 4 or 24 h was not sufficient for induction of suppressive cells (data not shown).

The background PFC numbers in the Con A-pre-treated cultures were in most cases elevated, possibly indicating a helper T cell induction resulting from a secondary B lymphocyte activation (Tables 1, 2, 3). Cytotoxicity did not seem to be the cause of suppression since cell viability was unaffected (Tables 2, 3).

In a second set of experiments, various concentrations of the hormone were tested for the induction of suppressor cells. A slight inhibitory activity was noted with pre-culture of cells in 500 IU/ml of hCG whereas 250 IU/ml was ineffective (Table 2). As shown in Fig. 1, a ratio of pre-cultured cells: fresh cells of 1 : 20 was required to induce a significantly suppressed PFC response, where IgM, IgG and IgA responses were found to decrease roughly in parallel.

TABLE 2

Suppressive effect of different hCG concentrations

Fresh autologous lymphocytes	48 h pre-incubation	Mitogen	PFC/culture IgM	% Suppression [a]	Survival ($\times 10^6$)
4×10^6	10^6	0	87		1.0
		PPD	6125	0	1.3
4×10^6	10^6 ConA	0	131		1.2
		PPD	437	95	1.8
4×10^6	10^6 hCG 2000	0	131		1.2
		PPD	2406	63	1.6
4×10^6	10^6 hCG 500	0	218		1.4
		PPD	4812	24	1.4
4×10^6	10^6 hCG 250	0	87		0.9
		PPD	6562	−7	1.4

Human peripheral blood lymphocytes were cultured in the presence of 50 μg Con A/ml, various concentrations of hCG or devoid of pre-activating substance. After 48 hours the cells were harvested and 10^6 pre-cultured cells were mixed with 4×10^6 autologous fresh lymphocytes. The admixture was cultured in the presence of 100 μg PPD/ml and harvested on day 6. Results represent PFC/culture.

[a] Background subtracted.

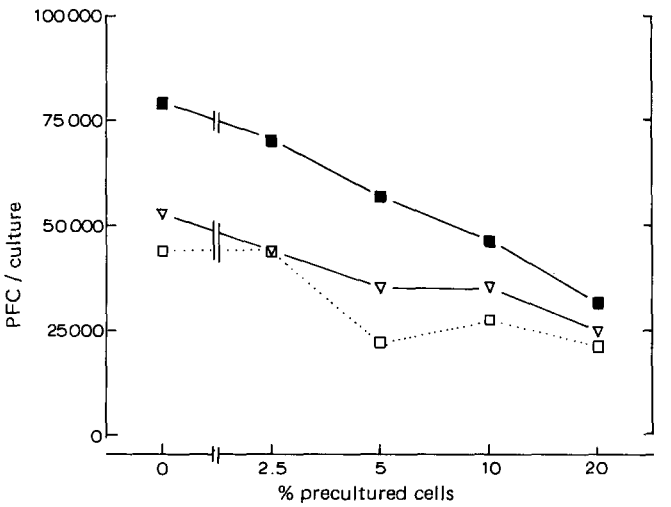

Fig. 1. Suppressive effect of hCG (2000 IU/ml) induced cells on fresh autologous PPD (100 μg/ml) stimulated lymphocytes. hCG-induced cells were mixed in different ratios after 48 h of pre-culture with the fresh cells to a final concentration of 10^6 cells/ml (5×10^6 cells/flask) and harvested on day 7. Results represent PFC/culture. ■, IgM; □, IgG; ▽, IgA.

TABLE 3

Suppressive effect of hCG-induced column-purified T cells

Substance used for preactivation	Mitogen	Unseparated cells							B cell-depleted cells						
		PFC/culture			% Suppression [a]			Survival $\times 10^6$	PCF/culture			% Suppression [a]			Survival ($\times 10^6$)
		IgM	IgG	IgA	IgM	IgG	IgA		IgM	IgG	IgA	IgM	IgG	IgA	
0	0	175	437	393				2.2	87	656	612				2.4
	PPD	38 500	24 500	23 185	0	0	0	2.0	35 875	21 000	18 375	0	0	0	2.4
Con A	0	87	437	1 662				2.0	131	218	1 575				2.0
	PPD	525	875	1 968	99	98	99	2.6	1 531	1 312	2 187	96	95	97	2.0
hCG	0	87	218	393				2.8	131	656	1 312				2.8
	PPD	21 875	6 125	12 301	44	76	49	3.2	21 875	15 750	10 500	40	26	49	4.0

Human peripheral blood lymphocytes, unseparated or column purified, were cultured in the presence of 50 μg Con A/ml, 2000 IU hCG/ml or devoid of pre-activating substance. After 48 h the cells were harvested and 10^6 pre-cultured cells were mixed with 4×10^6 autologous fresh lymphocytes. The admixture was cultured in the presence of 100 μg PPD/ml and harvested on day 6. Results represent PFC/culture.

[a] Background subtracted.

hCG-exposed purified T cells exert suppression

In the majority of suppressor cell systems hitherto described, T cells have been implicated as effector cells (see Möller, 1975). However, B cells and macrophages have occasionally been suggested as the cellular source of suppression (Persson, 1977). To elucidate the origin of hCG-induced suppressor cells, nylon-wool column cells, highly depleted of B cells and macrophages, were tested for suppressive capacity. In the unseparated cell suspension 29% of the cells were of B cell origin and in the nylon-wool column-purified cell suspension they constituted 2% as detected using a direct immunofluorescence method. A marked induction of suppressor cells was found in the 'purified' T cell suspension (Table 3), thus indicating a true T cell origin of suppressor cells.

Target for hCG-induced suppressor cells

Table 4 demonstrates that cells from the hCG stimulated cultures were suppressive when re-cultured with fresh autologous cells subsequently stimulated with PPD, PHA, LPS or PWM. This indicates that the target cell may be of T helper cell origin. In order to determine whether hCG-induced suppressor cells could act directly on B lymphocytes, their suppressive capacity was tested on EBV-stimulated cells, since this virus is a direct B cell activator. No suppressive effect was observed in the EBV-activated cell cultures by hCG-induced lymphocytes (Table 4).

TABLE 4

hCG- and con A-induced suppression. A comparison between different mitogens

Mitogen	No. of experiments	Mean suppression (%)					
		Con A			hCG		
		IgM	IgG	IgA	IgM	IgG	IgA
PPD	9	78	90	85	48 [a]	40 [b]	55 [c]
PHA	3	65	53	73	66	45	60
LPS	1	nt [d]	nt	nt	72	65	nt
PWM	1	tf [e]	77	99	tf	77	64
EB	3	75	68	84	−2	7	2

Human peripheral blood lymphocytes were cultured in the presence of 50 μg Con A/ml, 2000 IU hCG/ml or devoid of pre-activating substance. After 48 h the cells were harvested and mixed with 4×10^6 fresh autologous lymphocytes. The admixture was cultured in the presence of PPD (100 μg/ml), PHA (0.1 μg/ml), LPS (100 μg/ml), PWM (20 μg/ml) or in the presence of EBV (0.5 ml/10^6 cells in RPMI-medium supplemented with 10% FCS). The cells were harvested on days 5–7. Results represent mean suppressive effect of the different Ig classes.

[a] Range 14–73%, S.E. ±5.5; between groups a and b, $P > 0.05$ = n.s.; between groups a and c, $P > 0.05$ = n.s.

[b] Range 20–60%, S.E. ±5.6; between groups b and c, $P > 0.05$ = n.s.

[c] Range 33–87%, S.E. ±6.4.

[d] nt = not tested.

[e] tf = technical failure.

DISCUSSION

Human chorionic gonadotropin is produced in large amounts by the trophoblastic cells of the placenta throughout gestation. Up to the 6th–8th week of pregnancy it exerts a necessary luteotrophic effect, but after the luteoplacental shift its role is less clear. Several reports during the last decade have indicated that hCG has an immunoregulatory role and in vitro studies have demonstrated the ability of hCG to suppress a mitogen-induced proliferation of both T and B lymphocytes (see Introduction). This might to a large extent explain the necessity of a continuous production of hCG after the 8th week of gestation. The doses of hCG required to induce suppression have been exceedingly high (Beck et al., 1977) in comparison with mean serum values during pregnancy. However, the local concentration of hCG surrounding the trophoblast has been estimated to exceed 10 000 IU/ml of interstitial fluid (Braunstein et al., 1973), and in a recent study we have demonstrated that retroplacental lymphocytes, collected prior to legal abortion, were significantly suppressed in their mitogen-induced responses as compared to peripheral blood lymphocytes (Fuchs et al., 1977). This could possibly reflect the above mentioned concentration gradient.

The mechanism by which hCG might exert an immunoregulatory role, remains to be established. We have previously demonstrated hCG-induced suppressor T cells in the mouse (Fuchs et al., 1980). The present study indicates that the hCG preparations used have the capacity of inducing human T lymphocytes (Table 3), which in turn are competent to depress a mitogen-induced, polyclonal, T helper cell-dependent, antibody response of the IgM, IgG and IgA classes (Table 4). The hCG concentrations required to induce suppressor T cells are comparable to the high in vivo retroplacental concentrations of the hormone. The results indicate that the hCG-induced suppressor cells are of T cell origin (Table 3) since pre-cultured T cell-enriched lymphocytes exerted a suppressive effect indistinguishable from that of unseparated cells. These results are in accordance with those of Murgita et al. (1977) who, in the mouse system, found an enriched suppressor activity in α-fetoprotein-induced 'purified' T cells. The background PFC numbers in the Con A pre-treated cultures were elevated indicating an induction of helper T cells, which in turn activated a B-cell population. By using PPD, PHA, LPS and PWM stimulators as compared to EB virus, a direct B-cell activator (Bird and Britton, 1979), the intermediary effect on a prospective helper T cell target could be evaluated. Since no hCG-induced suppressive activity could be detected in the EBV stimulated cultures, this would indicate that the target cell for the hCG-induced suppressor cell is of T cell origin.

The mechanism by which hCG induces suppressor T cells is not yet clear. Normally, lymphocytes are activated via receptors localized in the surface membrane of the cell (Vidal-Gomez et al., 1978). However, Siebers and co-workers (1978) reported that human lymphocytes were not endowed with cell surface hCG receptors. Since suppressor T cells may constitute a minute fraction of the entire T cell population, a preferential binding of hCG to suppressor cells would not be detected in this model. Furthermore, the number of cell surface receptors does not necessarily parallel the biological effects induced by hormones (Fauci et al., 1980), and it has also been claimed that hormones internalize receptors when entering the cell, thus diminishing cell surface binding (see Kolata, 1978).

Polypeptide hormones, e.g. prolactin, human growth hormone and gonadotropins,

have also been reported to exert their long-term effects on cellular growth and metabolism by an intracellular mode of action. Whether a cell surface or intracellular triggering mechanism is involved in hCG induction of suppressor T cells is at present unknown. T lymphocytes from human newborns have been shown to exert a suppressor activity on mitogen or alloantigen-induced proliferation of adult lymphocytes (Olding and Oldstone, 1974). These suppressor T cells have been shown to carry receptors for the Fc part of IgG (Tγ cells) (Oldstone et al., 1977; Durandy et al., 1979). However, it has also been claimed that T lymphocytes bearing receptors for the Fc part of IgM (Tμ cells) may occasionally exert a suppressor effect on B cell maturation (Hayward and Lydyard, 1978). Two different cell populations might therefore be involved. It would in this context be tempting to assume that hCG, after having passed the placental barrier, would be responsible for the induction of cord blood suppressor T cells. However, since the concentrations or hCG in cord blood range from 0.01 to 0.1 IU/ml (P. Eneroth, personal communication), corresponding to a local concentration of 1–10 IU/ml on the fetal side of the placenta, if values are extrapolated according to the estimated relationship of blood/placental concentrations in the mother, this mechanism does not seem plausible. At least two different in vivo mechanisms must therefore be involved in the induction of human suppressor T cells, one of which might be dependent on hCG.

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