The effect of preparations of human chorionic gonadotropin on lymphocyte stimulation and immune response

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Summary. Optimal concentrations of human chorionic gonadotropin (HCG) enhance the uptake of tritiated thymidine ([H3]TdR) by nu/nu and BALB spleen cells maintained in tissue culture. The enhancing effect of HCG is found both with crude and with pure hormone. At higher concentrations the uptake of label is inhibited. BALB spleen cells transformed by Phytohaemagglutinin P (PHA) incorporate less label in the presence of crude HCG. This inhibition is not found with pure HCG. It is assumed that the inhibitory effect on T cells is due to a contaminant, whereas the enhancing effect on B cells may be due to the hormone itself.

Crude HCG has the ability to influence the immune response of murine spleen cells elicited in vitro and in vivo against sheep red blood cells (SRBC). At low concentrations the crude hormone inhibits the immune response, while higher concentrations increase it. Appropriate amounts of the crude hormone induce an immune response in vitro against SRBC by nu/nu spleen cells.

The daily administration of 200 iu/100 g i.p. of crude (2810 iu/mg) HCG suppresses the overt signs of an arthritic syndrome induced in Long–Evans rats by a mycobacterial adjuvant. In the Sprague–Dawley strain, where the arthritic syndrome is much more pronounced, a daily dose of 4000 iu/100 g of crude HCG considerably attenuates the swelling of the hind-feet of adjuvant arthritic rats. No effect is observed when pure HCG (10,000 iu/mg) is applied in the same doses to Long–Evans adjuvant arthritic rats.

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INTRODUCTION

HCG is thought to play a role in preventing the rejection of the foetus in the mammalian uterus (Beer & Billingham, 1971, 1974). Some preparations of HCG have been shown to affect the stimulation of lymphocytes in vitro. Some authors (Kage & Jones, 1971; Han, 1974; 1975) have attributed an inhibition of PHA-induced stimulation to the hormone itself whereas others have incriminated an impurity in the HCG preparation (Gundert, Merz, Hilgenfeldt & Brossmer, 1975). Inhibition of the mixed lymphocyte reaction by HCG preparations has also been reported to be due to a contaminant in the crude preparations (Caldwell, Stites & Fudenberg, 1975). These authors found that purified HCG enhanced the DNA synthesis of unstimulated lymphocytes. In the study described here an attempt has been made to correlate the effects of HCG preparations on the [3H]-labelled thymidine incorporation of cultured lymphocytes with effects on immune responses, as well as with the effect on an experimental arthritic in rats, susceptible to dexamethasone (Newbould, 1968).

MATERIALS AND METHODS

HCG

HCG was obtained from SIGMA (U.S.A.). The lyophilized material had a sp. act. of 3200 iu/mg. In its powder form, the sp. act. of the substance was 4000 iu/mg. These two preparations will be labelled 'crude'. A purer material (12,000 iu/mg) was purchased from A.B. LEO (Sweden).
No more than about 4000 iu of crude lyophilized HCG can be used in spleen cell cultures in vitro over a period of 4 days, in the absence of serum, without cytotoxicity (trypan blue exclusion). This cytoxicity is probably due to the presence of phosphates. In its powder form, the crude hormone was non-toxic, as was the purified hormone. This absence of toxicity was also found by Teasdale, Adcock, August, Cos, Battaglia & Naughton (1973).

**Animals**

BALB mice and Charles River white mice were purchased from Charles River (France), and nu/nu mice were obtained from Bomholgaard (Denmark). The following rat strains were purchased from Charles River (France), C-D, Fisher, Wistar and Long–Evans. The strain IOPS-OFA Sprague–Dawley was obtained from IFFA-CREDO (France).

**Chemicals**

Lyophilized phytohaemagglutinin P (PHA) obtained from Difco was employed at a final dilution of 1:1000. Concanavalin A (Con A) obtained through Sigma was used at a final concentration of 1 µg/ml. Dexamethasone was prepared at a concentration of 20 µg/ml in phosphate-buffered saline (PBS).

**Spleen cells**

Spleen cells were obtained from BALB mice and nu/nu mice. The cells were washed in Hanks's solution, cultured in RPMI 1640 medium at 37°C, under 5% CO₂, in erect tissue culture tubes (Falcon), without serum, except when stated. A concentration of 1 x 10⁶-5 x 10⁶ cells in 1 ml medium was used.

**DNA and RNA synthesis**

(H³-labelled methyl) thymidine (5 Ci/mm) at a final concentration of 1-5 µc/ml and C¹⁴-labelled uridine (50 mCi/mm) at a final concentration of 0.1 µc/ml were added to the medium. After incubation the cells (1 x 10⁶-5 x 10⁶ cells/ml medium) were precipitated with 10% cold TCA, washed five times on millipore filters with 5% cold TCA, the filters dried and the radio-activity determined in a liquid scintillation counter.

**Spleen cell sensitization**

Mice were injected i.p. or i.v. with 1 x 10⁶ SRBC. Booster inoculations were given 21 days later. In in vitro experiments, spleen cells were sensitized by addition of 5 x 10⁶ SRBC in 50 µl to 5 x 10⁶ spleen cells in 1 ml culture medium. The cells were washed in Hanks's solution and cultured in RPMI 1640 medium in the presence of 10% foetal calf serum and 1% streptomycin and penicillin, under 5% CO₂ atmosphere.

**Antibody-forming cell assay**

The haemolytic plaque assay of Jerne & Nordin (1963) was used.

**Experimental arthritis**

The arthritic syndrome was induced by a deep injection at the base of the tail of 0.05 ml of a sterile suspension of the inducer. The inducer was prepared by mixing equal volumes of light mineral oil 400-5 with a suspension of 5 mg/ml M. tuberculosis H37 RA Difco. The preparation was mixed in a vortex and briefly sonicated. After the inoculation of the inducer, the swellings of the hind-paws of arthritic rats were evaluated through visual inspection. This method was used when large numbers of animals were under experimentation. In addition, some strains responded only poorly to the inducer which made measurements of the thickness of the paws unrewarding. In more accurate studies with strains of rats that responded well to the inducer, the thickness of the paws was measured at the joint by a home-made mechanical device which amplified the dimensions 6-6-fold.

Rats of 80–100 g were used. Daily i.p. administration of the substances started on the day of inoculation of the arthritic inducer and usually continued for 15 days. The weight of the animals was recorded every 4 days and the volume of the inoculated fluids readjusted to maintain the appropriate dosage.

**RESULTS**

**Effect of pure and crude HCG on spleen cells stimulated by PHA or Con A**

High doses of pure HCG (800 and 1600 iu) were added to spleen cell cultures in the presence of various concentrations of Con A and PHA (Fig. 1). Pure HCG did not affect the incorporation of [³H] TdR into lymphocytes stimulated by PHA or Con A. Crude HCG decreased the incorporation of the DNA-precursor into PHA-stimulated cells and this depression of DNA synthesis was dose-related (Fig. 2).
Effect of various HCG preparations on DNA- and RNA-synthesis in spleen cells

Figure 3a and b shows the effect of crude HCG on $[^{14}C]$U and $[^{3}H]$TdR incorporation into BALB spleen cells. Fig. 3c establishes the similarity of the effect of crude HCG on spleen cells originating from both BALB and nu/nu mice. Fig. 3d shows the effect on BALB spleen cells, obtained with pure HCG. Crude preparations of HCG enhance the uptake of $[^{3}H]$TdR and $[^{14}C]$U by BALB spleen cell cultures. A similar effect was obtained with a pure preparation of HCG and spleen cells of nude mice. The possibility that the mere adjunction of protein to the cultures induced an increased incorporation of $[^{3}H]$TdR at high hormonal concentrations is unlikely because further increases in concentration of the hormonal preparations resulted in a sharp inhibition of DNA synthesis. Also, murine thymocytes cultured under the same conditions showed no increase in DNA synthesis, at any concentration of HCG (results not shown).

The two- to three-fold increase of $[^{3}H]$TdR uptake by B cells observed in the presence of optimal concentrations of HCG was paralleled by a slight increase from 2-4% (700 cells counted) in the number of blast cells present in the cultures. To substantiate further the effect of the hormonal preparation on lymphocytes, the immune response of murine spleen cells was analysed in a Jerne test.

**In vitro primary response in the presence of crude HCG**

Spleen cells obtained from Charles River white mice were cultured with SRBC and the number of specific IgM-secretory cells estimated on day 4 (Fig. 4). The crude HCG inhibited the response at a 10 iu/ml
concentration while higher concentrations did not. At very high doses complete suppression was seen. There was no general cell death (as shown by a dye exclusion test) during the experiment and the number of dye-excluding cells was similar in the HCG-treated cultures and the controls.

**Time of action of crude HCG**

A more detailed study of the effect of crude HCG on the number of cells engaged in a primary IgM response revealed that the inhibitory action of crude HCG occurs early, while higher doses may have an enhancing effect at later times (Fig. 5). The number of IgM-secretory cells was low on the 3rd day, reached a maximum on the 4th and declined sharply on the 5th day. Low doses of crude HCG decreased the number of secretory cells between days 3 and 5 of culture to levels less than that in unstimulated controls. High doses of the HCG preparation increased the number of secretory cells on day 5 of culture.

**Tertiary in vitro response**

The inhibition of the immune response is not restricted to unsensitized spleen cells. Spleen cells originating from sensitized mice respond identically to a tertiary *in vitro* immunization in the presence of crude HCG. Fig. 6 illustrates the inhibitory effect of the crude hormone on plaque-forming cells using both the indirect and the direct Jerne test. The inhibitory action of the hormone extended over a broader range of concentrations. Throughout these experiments it was apparent that the effect of crude HCG suggests an 'all or none' response. This was most obvious when the *in vitro* immune response was analysed with 'borderline' concentrations of HCG.

**Potentiation by crude HCG of the in vitro immune response by B cells**

Spleen cells from nu/nu mice respond very poorly to antigens such as SRBC (Wortis, 1971; Pantelouris, 1971). A response to SRBC was obtained with high doses of crude HCG (Fig. 7). Both direct PFC (day...
HCG on lymphocyte stimulation

Figure 4. In vitro primary response of Charles River murine spleen cells to SRBC in the presence of crude HCG. Crude HCG was added at the time of sensitization with the antigen, and the IgM response analysed on day 4. The analysis of each hormone dilution was done in duplicate, and the two values obtained are reported.

4) and indirect PFC (day 9) developed in the presence of HCG ranging from 250–2000 iu/ml.

Inhibition by crude HCG of the murine in vivo response

Repeated i.v. inoculations of crude HCG inhibits the immune response in vivo analysed 4 days after immunization (Fig. 8). Single doses have only a minimal influence on the outcome of the immune response (data not shown). Also the variability inherent in experiments with biological material makes this intermediary inhibitory response in vivo difficult to pinpoint.

Induction of the arthritic syndrome

Various strains of rats showed a different susceptibility to the arthritic inducer used (Fig. 9). The evaluation of the development of the syndrome was qualitative, with periodic visual inspection of the two hind-paws of each animal. Most susceptible was the Sprague-Dawley strain. The arthritic syndrome appeared very early and almost all rats showed the syndrome in an extremely pronounced form. The Fisher strain responded in a similar way but later in time. Much less susceptible was the Long-Evans strain.

Figure 5. Effect of crude HCG on IgM-response on days 3 (a) 4 (b) and 5 (c). In vitro primary response of Charles River murine spleen cells sensitized with SRBC. HCG powder was added with SRBC to spleen cells, and the IgM-response analysed on days 3, 4 and 5. The analysis of the response on each day for each dilution was done in duplicate, and the two values obtained are shown.
strain. The Wistar and C-D strains were similar to the Long-Evans strain in their response to the inducer. The five strains of rats used could thus be divided into two distinct groups showing different responses to the arthritic inducer. For evaluation of the effect of crude HCG on the arthritic syndrome, a representative strain of each group was used.

Effect of crude HCG on the experimental arthritic syndrome in Long-Evans rats

Long-Evans rats responded poorly to the arthritic inducer used. The poor development of the syndrome and the low relative number of arthritic paws observed in each experimental group made measurements of the changes in thickness of the paws impracticable and visual inspection was preferred. Daily doses of the crude HCG suppress the appearance of the syndrome. This suppression was dose related (Fig. 10). This demonstration of a suppressive effect of crude HCG on an arthritic syndrome was repeated in a more susceptible rat strain.

Effect of crude HCG on the experimental arthritic syndrome in Sprague-Dawley rats

The virulence of the syndrome in the Sprague-Dawley strain allowed a quantitative evaluation of the effect of crude HCG of which one single high dose was applied.

Crude HCG significantly suppresses swelling of the hind paws of adjuvant-arthritic rats (Fig. 11).

Figure 6. Effect of crude HCG on in vitro tertiary response analysed on day 9. Mice were twice sensitized with SRBC in vivo before being analysed for an in vitro response. Lyophilized HCG was added at the time of the sensitization in vitro. The IgM response and the IgM plus IgG response were analysed on day 9. The individual values are reported.

Figure 7. Effect of crude HCG on primary immune response in vitro of nu/nu spleen cells. Powder HCG was added with SRBC on day 0 and IgM-secretory cells were recorded on day 4. The total response was analysed in duplicate on day 7. Vertical bars represent the two values obtained per dose of HCG applied.
Dexamethasone, a known inhibitor of arthritis, was included as a control and inoculated at a dose of 10 μg/100 g. Its effect was identical to that of crude HCG. It is not shown in the figure for reasons of clarity, since the points are almost superimposable on those obtained from crude HCG-treated and control animals. Attempts at obtaining a remission of the arthritic syndrome through daily inoculations of crude HCG (4000iu/100g) after the syndrome was well established were unsuccessful (results not shown). Finally it was shown that the preventive

**Figure 8.** In vivo inhibition of IgM-response with HCG. Charles River common white mice were inoculated i.v. daily with various doses of lyophilized HCG (two animals/dose). On the third day of inoculation of the hormone, the mice were sensitized i.p. with SRBC. A fourth inoculation of HCG occurred one day later. The IgM-response was analysed 4 days after sensitization with the cellular antigen. The individual responses are reported in the figure.

**Figure 9.** Response of various rat strains to an arthritic inducer. The mycobacterial adjuvant (0.05 ml) was inoculated at the base of the tail of ten rats from each of five different strains. Visual inspection of the hind paws (twenty per experimental group) was made at various times after inoculation. The number of swollen or reddish hind paws was recorded. (■) Sprague-Dawley; (●) Fischer; (▲) C-D; (△) Wistar; (○) Long-Evans.

**Figure 10.** Effect of crude HCG on adjuvant arthritis in the Long-Evans strain. Crude HCG (2810 iu/mg) was administered daily i.p. to arthritic rats. The doses correspond to 32, 160 and 800 iu/100 g. Treatment was discontinued on the 17th day. Control (■ ■); 11 μg/100 g (▲ ▲); 57 μg/100 g (○ ○); 284μg/100 g (△ △).
Effect observed was not due to HCG itself but to a contaminant in the crude HCG preparation.

**Effect of pure HCG on an experimental arthritic syndrome**

For this purpose, the Long–Evans and Sprague–Dawley strains were used again. The administration of pure HCG to Long–Evans rats (Fig. 12) that were made experimentally arthritic could not prevent the appearance of the syndrome. Crude HCG administered in an equivalent dose calculated on an hormonal activity basis had this preventive effect. This failure to counteract the development of an arthritic syndrome with pure HCG was also found when the susceptible Sprague–Dawley strain was used. High daily doses of 2000–4000 iu of pure HCG had no effect on the outcome of the syndrome (results not shown).

The results demonstrate two effects of crude preparations of HCG on spleen cells: DNA-synthesis in B cells is promoted, whereas in helper T-cells it is repressed. The enhancing effect of crude HCG on DNA-synthesis of B cells was consistently observed. It was also found with a pure HCG preparation (LEO). However, this pure hormone is still not homogeneous. A higher purity, achieving 18,000 iu/mg can be obtained and a slight contamination...
with an LPS-like substance, perhaps in the urine, could account for the enhancing effect. The effect on T-cells is definitely due to a contaminant.

Lemonnier, Goussault & Bourillon (1973) have described a urinary glycopeptide and an oligosaccharide both of which have Phytohaemagglutinin P inhibiting activity. No inhibition of Con-A activity was observed. The glycopeptide may originate from red blood cells but the oligosaccharide has not been found in normal male urine. These authors (Lemonnier et al., 1972) attribute the activity of these compounds to binding to the phytoagglutinin. It is possible that our results may be explained by the presence of a similar PHA-binding substance, or it may be that the activity is due to a cell-binding substance.

The experiments performed indicate the presence in crude HCG of a substance that interferes with the blastogenesis induced by PHA. This interference takes place at the cellular level, because the addition of crude HCG to cultures stimulated by PHA produced no alteration in the blastogenesis induced by high and optimal concentrations of PHA as would have been expected had binding of the crude HCG to the lectin occurred. It is supposed that the binding of a substance in crude HCG occurs at a cellular site that is different from that of PHA and that this binding results in an inhibition of DNA synthesis. This hypothesis best accounts for the reduction in DNA synthesis observed in the presence of crude HCG at all doses of PHA applied, ranging from sub-optimal to excess, with a maximal incorporation maintained at the optimal dose of the mitogen. Other hypotheses such as binding of the inhibitor to the lectin, or competition for the same sites at the cellular membrane would result in a shift of the optimal response obtained by PHA. The inhibitory effect of the crude hormone at the doses applied is not manifest on Con A-treated spleen cells.

From our experiments, we conclude that the crude HCG preparations analysed contain at least two substances that influence helper T and B cells in opposite ways. The PHA-responsive cells are supposedly the T-cell subpopulation that fulfills a helper function in response to sheep red blood cells. The activity of these cells is suppressed by low concentrations of crude HCG. On the other hand, Con A has been considered either to provide a soluble factor that replaces the helper function of T lymphocytes (Dorries, Schimpl & Wecker, 1974) or else, is considered an index for the suppressive function of T lymphocytes (Gershon, Orbach-Arboys & Calkins, 1974). These cells are not much influenced by any of our hormone preparations.

The effect of crude HCG on the murine immune response against SRBC correlated with biochemical evidence showing that the crude hormone preparation activates or inhibits DNA synthesis of spleen cells according to dose.

At low doses crude HCG inhibits the immune response. This correlates with the inhibitory effect of the crude material on the incorporation of [³H]TdT by PHA-stimulated spleen cells. It was shown that this inhibition was due to a contaminant of the preparation (Caldwell et al., 1975; Gundert et al., 1975).

At high doses the 'pure' hormone enhances the immune response. The immune response is possible under those conditions, because HCG is able to trigger a response by bypassing the need for helper T cells. This was shown in HCG-treated nu/nu lymphocytes. The enhancement correlated with the increased DNA-synthesis of B cells. Pure hormone was available in only limited supplies and could not be used for the analysis of the immune response. It was, however, shown that the pure hormone stimulated the DNA-synthesis of B cells in concentrations at which an immune response is detected in nu/nu mice. It cannot, for the time being, be ruled out that small amounts of a B-cell mitogen contaminate even our best preparation of HCG. High doses are thus needed to detect their enhancing action on B cells in sophisticated in vitro tests. The immunoenhancing activity detected can thus only tentatively be ascribed to the hormone itself.

The fact that pregnancy sometimes provokes a remission of arthritic manifestations among humans (Richardson-Hill & Holley, 1974) prompted us to test the effect of the crude hormone preparation on an experimental arthritic syndrome.

Crude HCG preparations contain a substance that prevents the appearance of an experimental arthritic syndrome. In a very susceptible strain such as the Sprague–Dawley strain, a high dose of the preparation is needed and the effect is mostly noticed through a reduction in size of the swellings observed. In a more resistant strain such as the Long–Evans strain, the daily administration of an adequate amount of the substance succeeded in completely suppressing overt signs of the syndrome. This was not observed when pure HCG was used at
the same dose when calculated on its hormonal activity.

As long as isolation of this contaminant of the crude HCG preparation has not been achieved and its action has not been characterized in various immunological systems, it is not certain that the effects observed are related to one single substance. However, it seems likely that we are dealing with a single entity that suppresses expressions of cellular immunity in a unique way. By inference one could then conclude that a dysfunction of a cellular immune mechanism is at the roots of the arthritic diseases. In pregnancy, such a dysfunction would be corrected by the secretion of an inhibitory substance. Such a substance might be similar or identical to the one isolated from pregnancy urine by Lemonnier et al. (1973).

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