# DEVELOPMENT OF HYBRIDOMAS SECRETING ANTI - HUMAN CHORIONIC GONADOTROPIN ANTIBODIES

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**Running Title:** HYBRIDOMAS MAKING ANTI-hCG ANTIBODIES

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Human chorionic gonadotropin (hCG) is a hormone primarily synthesized and secreted by trophoblast. It is detectable in circulation in measurable amount within 8 to 10 days after fertilization of the egg[1,2]. Its level rises sharply in early phases of pregnancy, attaining a peak at 8 to 10 weeks of pregnancy, at which time approximately 4.6 mg of hCG is present per litre of serum[3]. Besides pregnancy, the hormone is also made by trophoblast and non-trophoblast tumours[4-6].

Antibodies against this hormone have wide utility in kits enabling the diagnosis of pregnancy. Likewise, these are also servicable in radioimmunoassays for quantitative estimation of the hormone. Qualitative and quantitative tests requiring anti-hCG antibodies are also useful in identification of hCG synthesizing tumours, their regression or progression during therapy and metastasis[7]. Anti-hCG immunization has also been conceived as an approach for control of fertility[8]. Antibodies against hCG bring about the termination of pregnancy in primates[9], and may eventually also serve for menstrual regulation.

For these and other applications, antibodies of high titre, specificity and reproducible characteristics are often required. Antibodies generated in animals by active immunization are heterogenous and variable from animal to animal. Furthermore, antibodies against a given antigen constitute only a small

fraction of the total globulins, even in the hyperimmunized animals, with the result that the immune sera have bulk of extraneous proteins with only a tiny part reacting specifically against the chosen antigen. These problems can be largely resolved by development of clones of hybrid cells producing exclusively antibodies against a given antigen. We describe here the successful development of a hybridoma secreting anti-hCG antibodies.

## MATERIALS AND METHODS

Animals: Balb/C male mice from either outbred or inbred stock were used for these experiments.

Reagents: Powdered RPMI-1640 medium was obtained from Grand Island Biological Company, U.S.A. N-2-hydroxyethyl-piperazine-N-2-ethane sulfonic acid (HEPES), fetal calf serum (FCS) where purchased from Flow Laboratories, U.K. Dimethyl sulfoxide (DMSO), hypoxanthine, thymidine, aminopterin, 2-mercaptoethanol were obtained from Sigma Chemical Company, St. Louis, Mo, U.S.A. 2, 6, 10, 14 - tetra methylpentadecane (Pristane) was obtained from Aldrich Chemical Company, Inc., U.S.A. Polyethylene glycol (PEG) 1550 was purchased from Serva, Heidelberg, Germany. Human chorionic gonadotropin (hCG; 10, 800 I.U/mg) was made available by Dr. Tsong of the Population Council, New York.

Culture and fusion media: The composition of the different media used for the maintenance of the myeloma cell line, fusion and selection of hyrbids was as follows:

i) RPMI medium: RPMI medium was prepared by dissolving in 1 litre of double distilled water, 10.4 g RPMI-1640 powder, 2.0 g $NaHCO_3$ and 2.38 g HEPES. 100 I.U. pencillin and 100 µg streptomycin per ml of the medium were also added. The medium was filtered through Millipore disc. (0.22 u pore size) and stored at 4°C.

ii) Growth medium: Growth medium was prepared by adding 20% sterile heat inactivated FCS to RPMI medium and stored at 4°C. Fresh glutamine at 20mM/ml medium was added if the medium was kept for more than 10 days before use.

iii) Fusion medium: It was prepared by adding sterile 0.5 ml of 2-mercaptoethanol ($5 \times 10^{-2}$M) to 500 ml of the Growth medium.

iv) HT 100 X solution: 130 mg hypoxanthine and 39 mg thymidine were dissolved in 100 ml of distilled water. To facilitate dissolution it was warmed to 45-50°C. It was sterilized by passing through Millipore disc (0.45 µ pore size) and stored at -20°C in 5.0 ml aliquots.

v) HAT 100 X solution: To prepare HAT 100 X solution 130 mg hypoxanthine, 1.76 mg aminopterin and 39 mg thymidine were dissolved in 100 ml of 10 mM NaOH, filtered through Millipore disc (0.45 µ pore size) and stored at -20°C in 5.0 ml aliquots.

vi) HAT and HT medium: HAT or HT medium was prepared by adding 1 ml of HAT or HT 100 X solutions to 99 ml of the Fusion medium.

vii) Polyethylene Glycol I (PEG I): 2 g PEG - 1550 was autoclaved in a glass tube at 15 pound pressure for 20 min. It was cooled to about 60°C and 2.2 ml RPMI medium and 0.6 ml DMSO were added, mixed and stored at 4°C.

viii) Polyethylene Glycol II (PEG II): 2 g PEG-1550 was autoclaved in a glass tube at 15 pound pressure for 20 min. 6.0 ml of RPMI medium was added to the cooled PEG, the solution mixed by vortexing and stored at 4°C.

Myeloma cell line and its maintenance: P3-NSI/1-Ag 4-1 (NSI) a non-secreting variant of P3-N63-Ag 8 (a cell line of Balb/C origin derived from MOPC-21) and which synthesizes only kappa Chain was used in these experiments. This was obtained from Dr. S.K. Kar, ILRAD, Nairobi, Kenya. The cell line was cultured in the Growth medium. To ensure that cells were as healthy as possible for fusion, they were kept in logarithmic growth phase for at least 4 days prior to fusion by subculturing them daily.

Immunization of mice: Balb/C male mice, 10-12 weeks old were immunized with hCG according to the schedule I & II as shown in Fig.1.

**Preparation of spleen cells:** Immunized mice having the highest titres of anti-hCG antibodies in sera were killed by ether anesthesia. Spleen was removed asceptically, placed in 5 ml of the Growth medium and cell suspension was made by teasing the spleen with a pair of curved forceps. Clumps and membrane fragments were allowed to settle and the resulting single cells were washed twice with RPMI medium by centrifugation at 800 rpm for 5 min. Cells were resuspended in RPMI medium, an aliquot diluted with equal volume of Trypan blue (0.1%), counted in hemocytometer and cell concentration was adjusted to $1 \times 10^7$ cells/ml medium.

**Preparation of macrophage feeder cells**

To prepare feeder cells, Balb/C male mice were killed by ether anaesthesia, drenched in 70% alcohol and peritoneum exposed. 5 ml of the cold RPMI medium was injected into the peritoneal cavity with the help of 18G needle, peritoneum tapped with fingers for 1 to 2 min and cells removed with the same needle. Washes from 2 to 4 mice were pooled, cells centrifuged at 1200 rpm and resuspended in 10 ml of the Growth medium. An aliquot of cells was diluted with an equal volume of Trypan blue (0.1%) and total nucleated cells counted in a hemocytometer. Cells were then diluted to $1 \times 10^5$ cells/ml and 1 ml of cell suspension plated per well in either 24 or 96 well plates (Coster, 205 Broadway Cambridge, U.S.A.). The feeder cells were incubated at 37°C in a humidified atmosphere of 5% $Co_2$, 95% air overnight before use.

Cell fusion and selection of hybrids: $5 \times 10^6$ myeloma cells were mixed with $5 \times 10^7$ splenocytes obtained from an immunized mice in a 10 ml plastic tube. The fusion was performed as per procedure outlined in Fig.2. 24 hrs after plating the fused cells, fusion medium from each well was replaced with 1.5 ml of the HAT medium. The HAT medium was changed thrice on alternate days. After that, cultures were watched for the growth of hyrbids and change in the pH of the medium. As soon as the growth was visible macroscopically or the medium turned acidic, supernatant was removed from each well with separate pipettes and analysed for the presence of anti-hCG antibodies. The cells from wells which were positive for anti-hCG antibodies were transferred to 60 x 15 mm tissue culture petri dishes (Falcon, 1950 Williams Drive, U.S.A.) for further propagation of the hybrids. A part of the hybrid cells were cloned immediately in order to keep in check the overgrowth of the non-producers. Hybrid cells were stored in liquid nitrogen.

Cloning of Hybrids: Hybrids positive for antibodies were cloned by limiting dilution technique. Hybrid cells were diluted in the HT medium and cloned in either 96 or 24 wells culture plates preincubated overnight with $1 \times 10^5$ feeder cells so as to get on average of 1 cell or 0.5 cell per well. Colonies became visible macroscopically by 7 to 10 days after plating and supernatants from well containing apparently a single colony were tested for antibody activity.

Preparation of ascites fluid: To obtain large amounts of monoclonal antibodies, Balb/C mice were given ip injection of 0.5 ml Pristane. One week later, healthy hybrid cells ($1\text{-}2 \times 10^7$) secreting antibodies were injected ip. When hybrids were grown as ascites (10 to 17 days), mice were killed by ether anesthesia and the ascites fluid was removed with the help of 18 G needle attached to 10 ml syringe. It was centrifuged at 1500 rpm for 10 min to remove cells. The ascites fluid was heat inactivated (56°C, 30 min), centrifuged at 10,000 g for 30 min and the supernatant was stored at -20°C in 0.5 ml aliquots.

Freezing of myeloma cells and hybrids: A 10% DMSO solution in the Growth medium was freshly prepared and cooled in ice. Cells to be frozen were centrifuged and resuspended at a concentration of $1.0 - 2.0 \times 10^6$ cells/ml in freezing medium. 1 to 1.5 ml of the cell suspension was distributed in 2 ml freezing vials (Wheaton Laboratory Products, Milville, U.S.A.) and the vials closed tightly. The freezing vials were kept at -70°C overnight in a well insulated plastic beaker with cotton gauze to allow slow cooling. Next day the vials were transferred into a liquid nitrogen tank (LR-40, Union Carbide, 8 Grafton Street, London).

Antibody titre: Anti-hCG antibody titres in the sera obtained from immunized mice or in the supernatants obtained from wells with positive growth of hybrids were determined by radioimmunoassay as described elsewhere[10].

Specificity studies: Using the raioimmunoassay systems, the binding of the anti-hCG antibodies secreted by hybrid cells was determined with $^{125}$I-hLH (WHO - Quality control Reagent, Batch No.8010) and $^{125}$I-hFSH (WHO - Quality control Reagent, Batch No.8010).

## RESULTS

Four fusion experiments were carried out. In three of them, mice were immunized according to Schedule I (Fig.1) each mouse at the time of the experiment had fairly good anti-hCG titres in the serum (Table I). The spleen cells obtained from these mice, however, generated hybrids which grew in HAT medium but none of the well had appreciable titre of anti-hCG antibodies (Fig.3, Schedule I). An alternate immunization protocol was followed (Schedule II, Fig.1). Spleen cells from mouse immunized thus, on fusion with NSI myeloma cells gave rise to 42 wells out of 48 plated, in which growth was detectable in the HAT medium. The supernatants from 10 of the wells had anti-hCG titre above 10% (Schedule II, Fig.3).

In the next step, cells of well No.15 with the highest antibody titres as well as hybrid cells from wells No.5,16 and 42 were cloned by limiting dilution technique in 96 and

24 well plates. Hyrbid cells from other wells as well as these 4 wells wese frozen in liquid nitrogen as stock. After cloning, growth of the hybrids was seen to variable extent in each case (Table 2). However, the anti-hCG antibodies were measurable only in $H_{15}$ clones; 110 out of 112 wells in which the hybrids grew were positive for anti-hCG antibodies. 40 of these were taken for further studies. All of them made antibodies with high hCG binding capacity ranging from 85 to 95%. The stock cultures of these 40 clones were frozen.

Specificity studies were carried out on cloned cells from 10 wells. The reactivity of the antibodies from these clones with radiolabelled hCG and the pituitary gonadotropins is given in Table 3. All of them had high percentage normalized binding with hCG ranging from 88 to 99.9%. The cross-reactivity of the supernatants from 8 wells with hLH was less than 4.5%. Two wells, however, had antibodies with higher binding with hLH i.e. well No.84 had 14.1% and well No.51 had 11.5%. As hCG shares a common $\alpha$-subunit with hFSH also, the cross-reactivity of the antibodies with this hromone was also tested. This was, however, negligible.

Cells from one of the wells ($P_{III}W_{80}$) were injected ip. into a Balb/C mouse as per the procedure in the methods. Ascites was developed and on day seventeen the intraperitoneal fluid was aspirated. A single mouse yielded 4.5 ml of the supernatant with a protein content of 39.7 mg/ml. The anti-hCG titres

were high, a binding of 30% of $^{125}$I-hCG was obtained with this supernatant at 4 x $10^6$ dilution. These are far beyond the titres ever seen or reported in animals even when hyperimmunized.

## DISCUSSION

The technique initially developed by Köhler and Milstein[11,12] to obtain hybrid cells by fusion of a cell making a defined type of antibody with a myeloma cell capable of continuous multiplication in a culture medium has opened out new avenues for preparation of highly specific antibodies against different types of antigens. Using this technique we have developed a number of clones committed to synthesis of anti-hCG antibodies. Only a small number of these have been characterised so far. Amongst the ten investigated, at least five subsets are detectable. One of them is highly specific for binding to hCG with negligible binding (below detectable limit) with hLH and hFSH. The second subset has high binding with hCG, devoid of binding with FSH and has low, below 2% binding with hLH. The third subset had 2.1% binding with hFSH, 0.3% binding with hLH as compared to 92.4% binding with hCG. The fourth subset had a slightly higher but still low e.g. 3.3% binding to hLH and no reactivity with hFSH. The fifth subset had 11 to 14% reactivity with hLH. It is obvious that antibodies from

these clones react with different portions and conformations of hCG. The subset with more appreciable cross-reaction with hLH will be binding to conformation shared by the two gonadotropins. The existence of clones highly specific to hCG and practically devoid of reactivity with hLH and hFSH points to the presence in hCG of conformations unique to it.

Each one of these clones is able to propogate not only in culture but importantly a large amount of antibody with very high titres can be obtained by injecting these cells into the mouse peritoneum. The product of a given clone would be of defined specificity and of consistant characteristics.

The antibodies from the presently described clones can have immediate utility as standard reagents for radioimmunoassays for hCG. Preliminary experiments have also indicated the ability of the antibodies from these clones to neutralize the biological activity of hCG in the mouse Leydig cells assay. Investigations are in progress to determine whether these antibodies alone or in mixture would retain potent neutralizing capability in vivo. In case, they are effective for termination of pregnancy, these can constitute readily producible material of standard quality for passive immuni$^{z}$ation procedures.

SUMMARY

A number of clones of hybrid cells have been developed by fusion of P3-NSI/1-Ag4-1 (NSI) myeloma cells with splenocytes of mice immunized with hCG. The binding characteristics of the products of ten clones with the three gonadotropins e.g. hCG, hLH and hFSH have been determined. Three clones make antibodies of high reactivity with hCG but negligible binding (below detectable limit of assay) with hFSH and hLH. The clones when injected in mouse peritoneum produce homogenous antibody of very high titre with consistent characteristics. One of their imminent applications is foreseen as standard reagents for estimation of hCG.

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## LEGENDS TO FIGURE

**Fig. 1.** A schematic representation of the immunization of Balb/C mice with hCG.

| | |
|---|---|
| CFA | Complete Freund's Adjuvant |
| IFA | Incomplete Freund's Adjuvant |
| Sc | Subcutaneously |
| ip | Intraperitoneal |
| iv | intravenous |

**Fig. 2.** Procedure followed for fusion of NSI myeloma cells with splenocytes obtained from mouse immunized with hCG.

**Fig. 3.** 50 µl of culture fluid from each of the 48 wells in which the fused cells were plated, was tested for its ability to bind hCG. Percentage normalized binding of $^{125}$I-hCG is calculated by the following formulation.

$$\text{Percentage Normalized Binding} = \frac{X - B}{Y - B} \times 100$$

X = Amount of $^{125}$I-hCG bound with test sample.

Y = Amount of $^{125}$I-hCG bound in presence of excess of standard anti-hCG antibodies.

B = Amount of $^{125}$I-hCG bound in presence of 50 µl of normal culture medium.

TABLE 1 - LACK OF CORRELATION OF CIRCULATING ANTI-HCG TITRES IN DONOR MICE OF SPLENOCYTES WITH FREQUENCY OF HYBRIDS POSITIVE FOR ANTIBODY

| | Schedule I | Schedule II |
|---|---|---|
| Anti-hCG Titre in immunized mice serum | 84, 144, 114* | 7.3 |
| Frequency of hybrids positive for antibody. | Nil | 24%** |

*Titres in 3 different mice used as donors of splenocytes.

**$\frac{\text{Wells positive for anti-hCG antibodies}}{\text{Wells with growth of hyrbid cells}} \times 100$

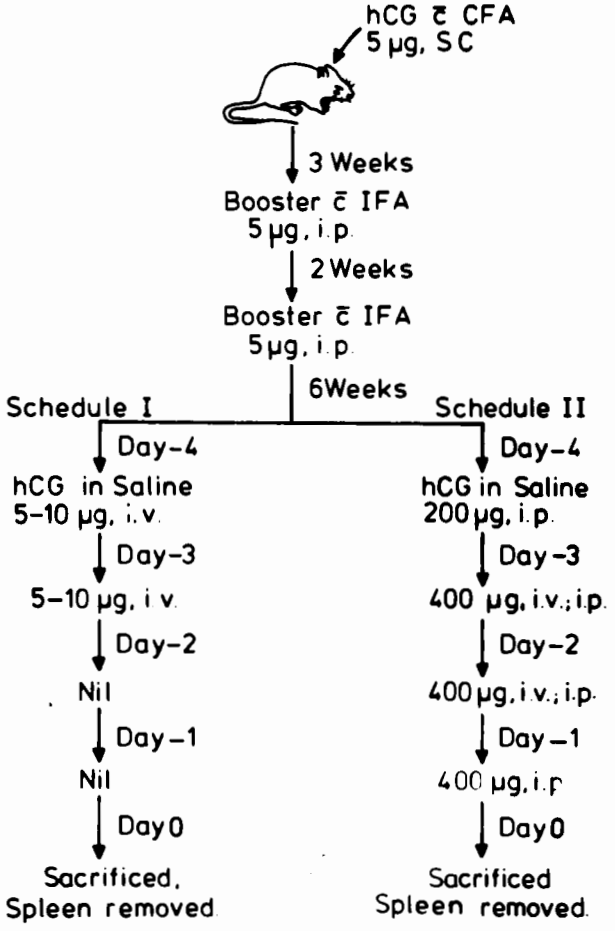
TABLE 2 - CLONING OF ANTI-HCG POSITIVE WELLS

| Well No. | No. of wells with growth/Total No. of wells plated | No. of wells +ve for anti-hCG antibodies |
|---|---|---|
| $H_5$ | 5/24 | - |
| $H_{15}$ | 112/144 | 110 |
| $H_{16}$ | 17/24 | - |
| $H_{42}$ | 20/24 | - |

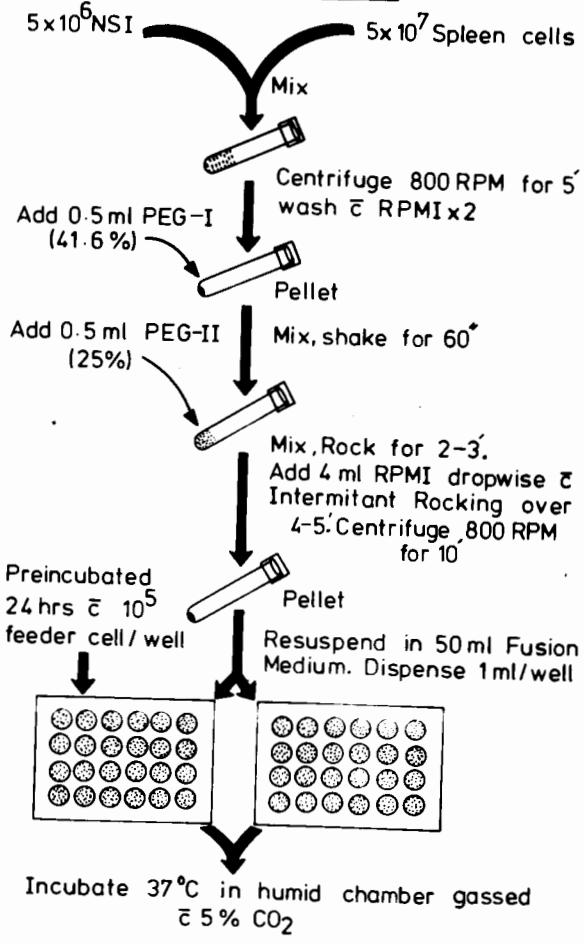
TABLE 3 - BINDING CHARACTERISTICS OF THE PRODUCTS OF CELLS FROM 10 WELLS CLONED FROM $H_{15}$

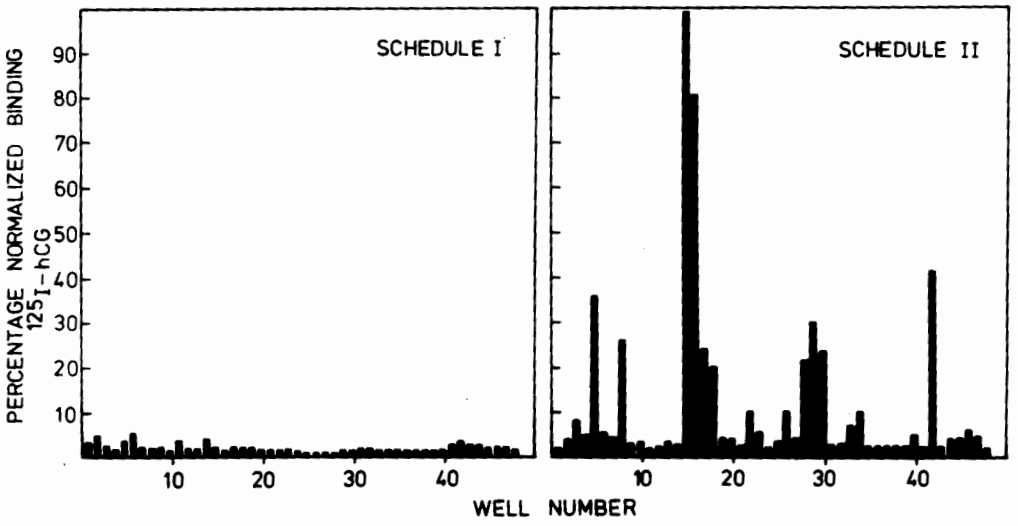
| WELL NUMBER | PERCENTAGE NORMALIZED BINDING $^{125}$IODINATED PEPTIDES | | |
|---|---|---|---|
| | hCG | hLH | hFSH |
| $P_{II}\ W_5$ | 99.9 | - | - |
| $P_{II}\ W_{15}$ | 92.4 | 0.3 | 2.1 |
| $P_{III}W_9$ | 94.4 | 3.3 | - |
| $P_{III}W_{67}$ | 92.6 | 1.6 | - |
| $P_{III}\ W_{68}$ | 95.8 | 0.8 | - |
| $P_{III}\ W_{80}$ | 97.8 | - | - |
| $P_{III}\ W_{81}$ | 95.7 | 2.0 | - |
| $P_{III}\ W_{96}$ | 88.0 | 1.4 | - |
| $P_{III}\ W_{84}$ | 99.8 | 14.1 | - |
| $P_{III}\ W_{51}$ | 94.31 | 11.5 | - |

## IMMUNIZATION SCHEDULES OF MICE




## FUSION PROTOCOL OF MYELOMA WITH SPLEEN CELLS




SCHEDULE I
SCHEDULE II
PERCENTAGE NORMALIZED BINDING
125I-hCG
90
80
70
60
50
40
30
20
10
10
20
30
40
10
20
30
40
WELL NUMBER