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A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor; partial characterization of the receptor

Warren J. Leonard, Joel M. Depper, Takashi Uchiyama, Kendall A. Smith*, Thomas A. Waldmann & Warner C. Greene

The Metabolism Branch, National Cancer Institute, NIH, Bethesda, Maryland 20205, USA and *Dartmouth Medical School, Hanover, New Hampshire 03226, USA

T-cell growth factor (TCGF or interleukin-2) is an inducible glycoprotein hormone of molecular weight 15,000 (ref. 1) synthesized and secreted by T lymphocytes following activation with antigen or mitogen[2,3]. TCGF is required for proliferation and expansion of T cells following antigen encounter[4,5] and to maintain them in long-term culture *in vitro*[6–9]. Full expression of the human immune response requires both the induction of TCGF synthesis and the formation of specific TCGF membrane receptors[10,11]. Monoclonal antibodies binding TCGF have been prepared[12,13]. In contrast, antibodies specific for the TCGF membrane receptor have not been identified, nor has the receptor been characterized. We have prepared a monoclonal antibody, termed anti-Tac[14,15], which appears to bind to the human membrane receptor for TCGF. In support of this, we now demonstrate that anti-Tac suppresses TCGF induced proliferation of T cells and blocks binding of radiolabelled TCGF to cells from a cloned human continuous T-cell line. Also we have partially purified and characterized the putative TCGF receptor. This receptor is a glycoprotein with a molecular weight (M_r) of 47,000–53,000.

Monoclonal anti-Tac was used in both ascites and Protein A–Sepharose purified[16] forms. Two human continuous T-cell (CTC) lines, CTC-2 and HUT–102B2, were used in these studies. CTC-2 is TCGF dependent and derived from the peripheral blood of a patient with a nonleukaemic cutaneous T-cell lymphoma. CTC-2 is a subclone of CTC-1 and has been in culture for more than 2 yrs. HUT–102B2 is a cloned CTC line established from a lymph node of the same patient[17]. In contrast to CTC-2, HUT–102B2 constitutively produces sufficient endogenous TCGF so that exogenous TCGF is not required. Cells from this clone express membrane TCGF receptors[18]. As recently reported by Poiesz and co-workers[19], HUT–102B2 cells contain and shed type C retrovirus. Cells from both CTC lines formed rosettes with sheep erythrocytes, reacted with OKT3 monoclonal antibody (a pan-T-cell-specific reagent)[20], and did not exhibit detectable surface immunoglobulin or Epstein–Barr nuclear antigen.

We initially looked at the effect of anti-Tac on TCGF-induced proliferation of CTC-2 cells. As shown in Fig. 1*a*, anti-Tac ascites markedly inhibited TCGF-induced ^{3}H-thymidine incorporation at final dilutions of 10^{-2}–10^{-4}, whereas equivalent concentrations of control ascites had no significant effect. Purified anti–Tac antibody also inhibited proliferation of CTC-2 cells, but purified monoclonal anti-Ia, though binding to these cells, had no effect (Fig. 1*b*). In agreement with these data, G. Bonnard and co-workers have independently demonstrated anti-Tac inhibition of TCGF induced proliferation of other human CTC lines (personal communication). In contrast, anti-Tac did not inhibit the growth of TCGF independent T-cell lines (CEM, HUT–78) or Epstein–Barr virus-transformed B-cell lines including a B-cell line derived from the same patient as the CTC lines.

All these experiments were performed in the absence of complement and anti-Tac did not produce cell death measured by short-term (4 h) ^{51}Cr -release assays or long-term (24–48 h) supravital dye exclusion[21] studies. In addition, the inhibitory effect of anti-Tac on proliferation of CTC-2 cells was reversible. CTC-2 cells precultured for 18 h with inhibitory concentrations of anti-Tac (10^{-3} dilution of ascites) incorporated thymidine in amounts equivalent to untreated cells following removal of cell-associated antibody by thorough washing and stimulation with TCGF.

We next investigated whether anti-Tac inhibited TCGF-induced proliferation by blocking the interaction of TCGF with its membrane receptor. ^{3}H-TCGF was prepared by phytohaemagglutinin induction of TCGF synthesis in a cloned human T leukaemia cell line (JURKAT)[22] in the presence of ^{3}H-lysine and ^{3}H-leucine. Radiolabelled TCGF produced in the absence of serum was purified by ultrafiltration and molecular sieve chromatography as previously described[10], and produced a single spot when evaluated by two-dimensional gel electrophoresis. As shown in Fig. 2, preincubation of HUT–102B2 cells with anti-Tac at concentrations greater than 3 μg ml^{-1} produced nearly complete blockade of purified ^{3}H-TCGF binding in experiments performed at 37 °C. In contrast, while binding to these cells, similar amounts of monoclonal anti-Ia did not alter ^{3}H-TCGF binding. The dose–response relationship

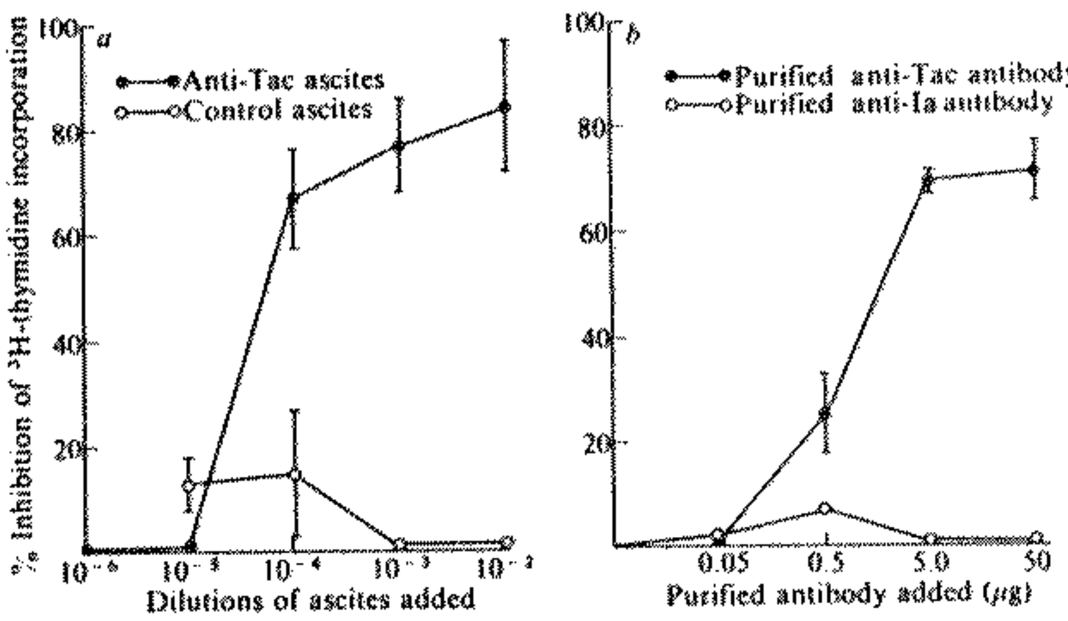

Fig. 1 Anti-Tac blocks TCGF induced proliferation of T cells from a human CTC line. CTC-2 cells were washed four times in a balanced salt solution and suspended at 0.5×10^6 cells per ml in RPMI 1640–10% heat-inactivated fetal calf serum. Aliquots (0.2 ml) of this cell suspension were distributed into wells of flat-bottom 96 well microtitre plates and preincubated with varying concentrations of antibodies for 30 min at room temperature. *a*, Data obtained with dilutions of heat-inactivated anti-Tac and control ascites (SPQC 11, from Dr M. Potter); *b*, data obtained with Protein A–Sepharose-purified anti-Tac and DEAE-purified monoclonal anti-Ia antibodies. Crude TCGF was subsequently added at a final concentration of 10% v/v. Triplicate cultures were incubated for 48 h at 37 °C in a humidified atmosphere containing 5% CO_2. Four hours before the end of culture, 1.0 μCi of ^{3}H-methyl-thymidine was added to each well followed by transfer of cells onto fibreglass filters with a multiple channel automated cell collector. Cell-associated radioactivity was measured by liquid scintillation. Data are expressed as % inhibition of the response obtained in cultures receiving TCGF alone (28,288 ± 784 c.p.m.).

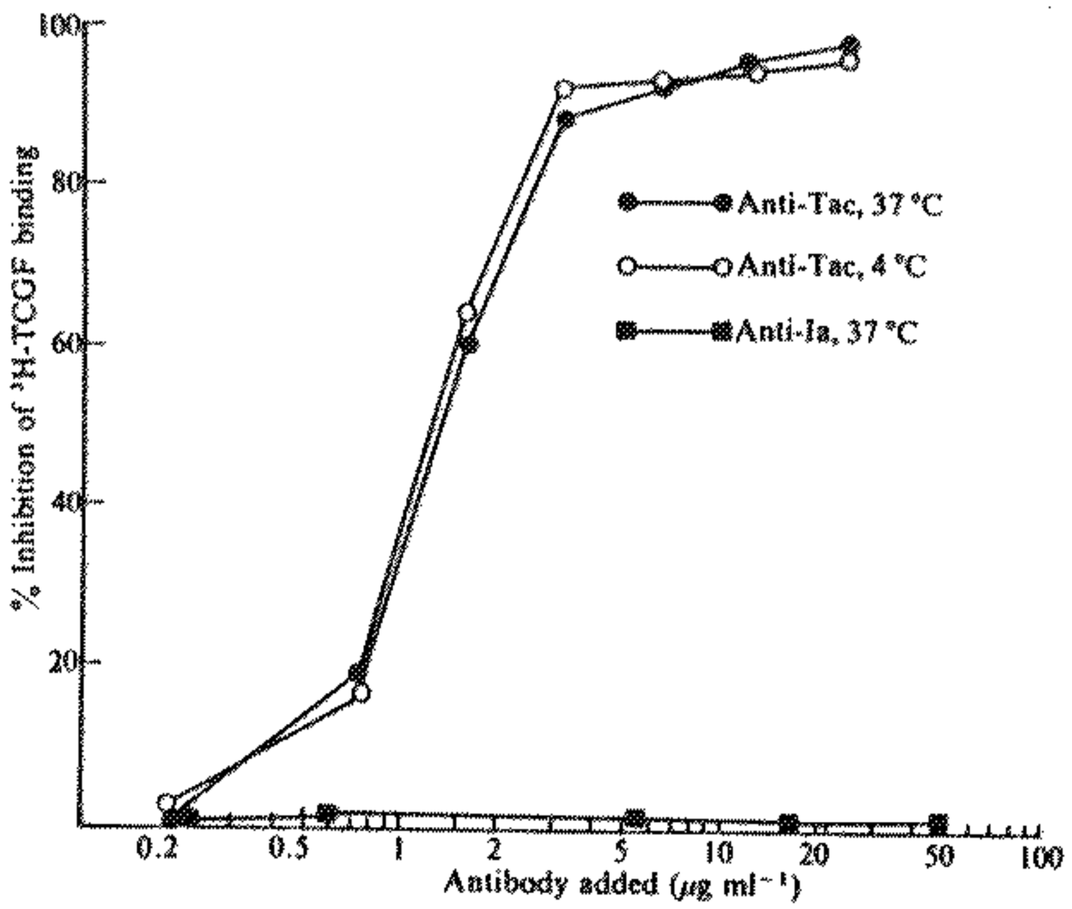

Fig. 2 Anti-Tac blocks the binding of ^{3}H-TCGF to HUT-102B2 cells. HUT-102B2 cells were washed three times in RPMI 1640 and resuspended at 1.1×10^7 cells per ml in RPMI 1640 containing 25 mM HEPES and 2 mg per ml bovine serum albumin. Cells and varying amounts of purified anti-Tac or anti-Ia antibodies were incubated together for 90 min at 37 or 4 °C in a rotating water bath. Following this preincubation, 50 pmol of purified ^{3}H-Leu, ^{3}H-Lys TCGF were added and the reaction was continued for an additional 20 min at 37 °C or 60 min at 4 °C. Cells were then pelleted, resuspended in 100 μl of RPMI 1640, and centrifuged (9,000*g* for 90 s) through cushions of 84% silicone and 16% paraffin oil to remove unbound ^{3}H-TCGF. Microfuge tube tips were excised, placed in liquid scintillation vials, and the cells solubilized by addition of 100 μl 1% SDS and 3 ml of liquid scintillation mixture followed by measurement of cell-associated radioactivity. Each experiment was performed in duplicate. Data shown are from two different experiments. A third experiment performed at 37 °C produced similar results.

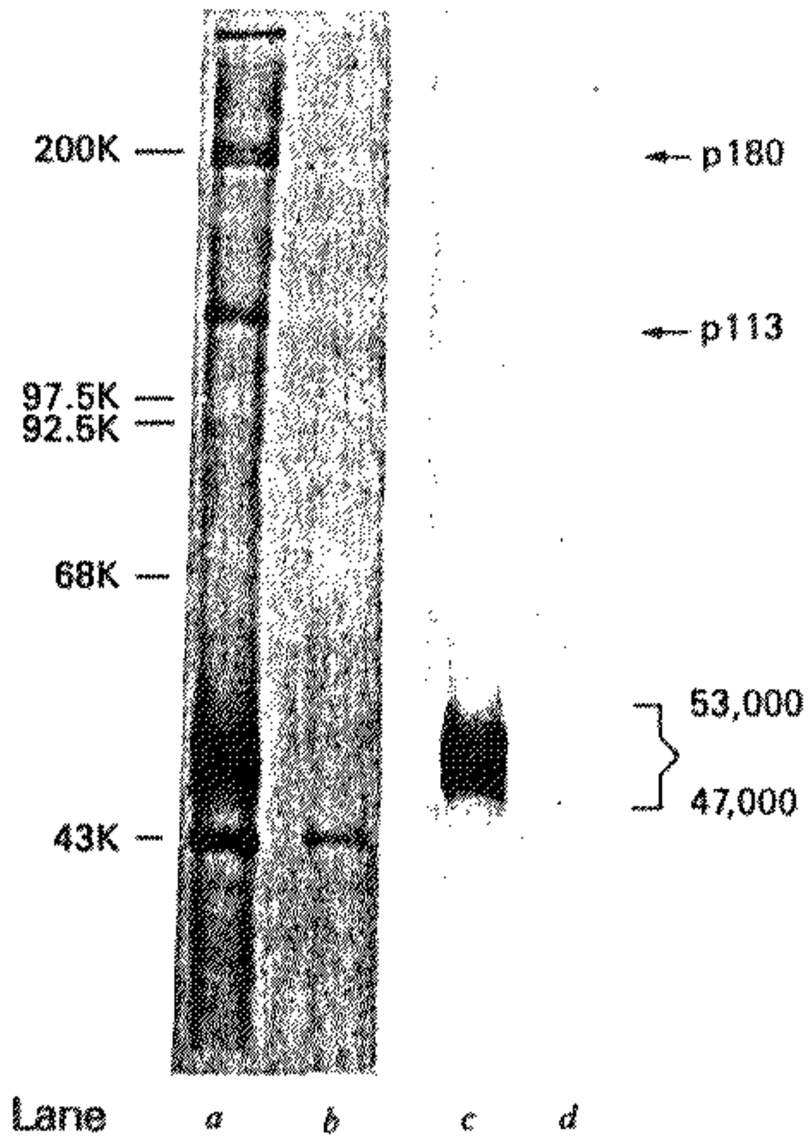

Fig. 3 Purification of the anti-Tac membrane receptor. Five million HUT-102B2 cells were washed twice in a balanced salt solution and suspended in 5 ml of methionine-free Selectamine medium (Gibco) containing 5% dialysed fetal calf serum. 0.3 mCi of NEN translation grade ^{35}S-methionine (>800 Ci mmol^{-1}) was added and the cells were cultured overnight at 37 °C. Cells were washed once and extracted in 0.14 M NaCl, 10 mM Tris *p*H 7.5, 1% Triton X-100, and 100 μg ml^{-1} phenylmethylsulphonyl fluoride (PMSF) for 30 min at 4 °C. The supernatant of a 60-min spin at maximum speed was then immunoprecipitated as follows. The supernatant was cleared once with 5 μl of control ascites (NS1, a non-secreting ascites provided by Dr Jay Berzofsky, NIH), 5 μl of RPC5 (a control IgG2a-k antibody, Litton Bionetics), and 50 μl of 10% w/v formaldehyde fixed Cowan I strain staphylococci and then once with 50 μl of the staphylococcal organisms alone. The supernatant was immunoprecipitated with 2–3 μg of protein A sepharose purified anti-Tac or UPC10 (a second control IgG2a-k antibody, Litton Bionetics) for 20 min at room temperature followed by a 20 min incubation with 20 μl of Cowan I strain staphylococci at room temperature. The immunoprecipitate was resuspended in a buffer containing 0.1% SDS, 0.5% NP40, 0.2% deoxycholate, 100 μg ml^{-1} PMSF and washed twice through the same buffer containing 1M sucrose. Final pellets were boiled in 1% SDS in the presence or absence of 0.1M dithiothreitol and analysed on 7.5% discontinuous SDS-polyacrylamide gels. Gels were intensified and fluorographed for 24 to 48 h at −195 °C. Analysis under reducing conditions is shown. *a* and *b* show the electrophoretic patterns obtained following immunoprecipitation of ^{35}S-methionine incorporated cells with anti-Tac and UPC10, respectively. The bands at 200,000 (200K) and 43,000 (43K) co-migrate with myosin and actin, respectively. Migration of molecular weight standards is shown on the left. The locations of p180, p113, and p50 are indicated on the right. *c* and *d* represent electrophoretic patterns obtained after immunoprecipitating surface-labelled cells with anti-Tac and UPC10, respectively. Approximately $5–10 \times 10^6$ cells were washed and suspended in 100 μl of a balanced salt solution and 1 mCi carrier-free ^{125}I was added. 4 μl lactoperoxidase solution (1U per 10 μl) and 7.5 μl H_2O_2 solution (0.03%) were then added and cells incubated for 4 min at room temperature. An additional 2 μl of lactoperoxidase solution and 7.5 μl H_2O_2 were added and cells incubated for an additional 4 min. Cells were then washed in a balanced salt solution and immunoprecipitated as described above.

for anti-Tac inhibition of TCGF-induced CTC proliferation and blockade of TCGF binding were similar in several experiments. As shown in Fig. 2, anti-Tac also inhibited ^{3}H-TCGF binding at 4 °C. These data argue that anti-Tac inhibition of TCGF binding is not simply secondary to receptor capping or co-capping with subsequent receptor endocytosis or shedding, because these rearrangements are largely inhibited at 4 °C (ref. 23).

Studies of ^{125}I-anti-Tac binding to HUT-102B2 cells at 20 °C revealed half saturation of available receptor sites within 3.75 min of anti-Tac addition. Next, ^{125}I-anti-Tac was incubated with HUT-102B2 cells at 4 °C in the presence of 0.1% sodium azide to block cap formation and energy-dependent endocytosis. After 1 h, steady-state binding was achieved. Excess unlabelled antibody was then added and reversible binding was demonstrated as 90% of the bound radiolabelled anti-Tac molecules were shown to dissociate. Glutaraldehyde fixed HUT-102B2 cells bound ^{125}I-anti-Tac in amounts slightly less than non-fixed cells. Preliminary Scatchard analysis[24] of anti-Tac binding with HUT-102B2 cells indicates ~100,000 high-affinity receptors per cell. This receptor number is 7- to 8-fold higher than the number of TCGF receptors reported by Robb *et al.*[10]. This difference may in part be accounted for by different experimental binding conditions and slightly different cell lines (our HUT-102B2 line is not 6-thioguanine resistant in contrast to that of Robb and co-workers). Anti-Tac had a K_d of $\sim 10^{-9}$ mol l^{-1} suggesting a much lower affinity than that reported for TCGF ($K_d \sim 10^{-12}$ mol l^{-1})[10]. Thus, the preincubation with anti-Tac before addition of ^{3}H-TCGF may have been critical to our ability to perform the successful competitive binding experiment (Fig. 2).

Finally, we have partially purified and characterized the membrane receptor recognized by anti-Tac. SDS-polyacrylamide gel electrophoretic analysis of this receptor from HUT-102B2 cells is shown in Fig. 3. In both reducing (shown) and nonreducing (not shown) conditions, two major proteins with molecular weights of approximately 113,000 and 47,000–53,000 were precipitated by anti-Tac antibodies from cells internally labelled

with ^{35}S-methionine (*a*). A third minor band with an M_r of approximately 180,000 was also identified. For convenience, we will denote these bands p113, p50, and p180, respectively. None of these bands was identified in immunoprecipitations using control UPC10 IgG2a-k antibodies (*b*). Further, similar bands have been found in immunoprecipitations performed on phytohaemagglutinin stimulated peripheral blood lymphocytes from a normal individual (data not shown), thereby providing evidence that none are related solely to viral products. To determine whether any of these proteins are located on the outer cell membrane, similar anti-Tac and UPC10 immunoprecipitations were performed using cells that were surface iodinated with the lactoperoxidase method (*c* and *d*). Only p50 was immunoprecipitated with anti-Tac. We therefore hypothesize that this protein represents the actual binding site for anti-Tac. This protein can be identified in immunoprecipitations from cells incorporated with ^{3}H-D-glucosamine (Fig. 4), thus demonstrating that it is a glycoprotein, as is usually the case for membrane receptors. The roles of p113 and p180 are unknown. One or both may be part of a theoretical receptor complex, and therefore coimmunoprecipitated because of strong hydrophobic interactions with p50. It is also possible that neither has any functional relation to the receptor identified by anti-Tac, but rather that they contain antigenic determinants that result in their recognition by anti-Tac.

In summary, we have prepared a monoclonal antibody which blocks the binding of purified ^{3}H-TCGF to human CTC cells and inhibits TCGF induced proliferation of CTC cells, and which we hypothesize binds to the TCGF receptor. The receptor recognized by the antibody is a glycoprotein of molecular weight ~47,000–53,000. In recent studies with human peripheral blood T cells, we have also found that anti-Tac inhibits (>80%) soluble or alloantigen induced T-cell proliferation as well as the generation of cytotoxic T cells in allogeneic cell cocultures. Further, anti-Tac suppresses immunoglobulin production in cultures of peripheral blood lymphocytes stimulated with pokeweed mitogen, a T-cell dependent polyclonal activator. Thus, anti-Tac may permit not only further characterization of the human TCGF receptor but in addition may allow analysis of the site(s) and mechanism of TCGF action in both the cellular and humoral phases of the human immune response.

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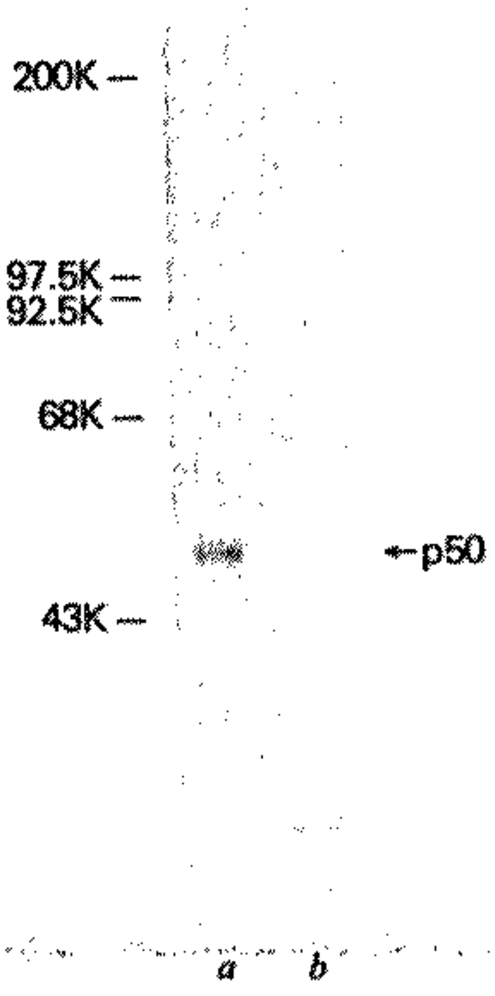

Fig. 4 The anti-Tac membrane receptor is a glycoprotein. 5×10^6 HUT-102B2 cells were washed once in a balanced salt solution and resuspended in RPMI media containing 5% fetal calf serum. Two hundred microcuries of D-[1,6-^{3}H(*N*)]-glucosamine HCl (NEN, 32.5 Ci mmol^{-1}) were then added and cells incubated for 4 h at 37 °C. Cells were washed once in balanced salt solution and then extracted with 0.14 M NaCl, 10 mM Tris-HCl *p*H 7.5, 1% Triton X-100, and 100 μg ml^{-1} PMSF for 30 min on ice. The extracts were then immunoprecipitated with anti-Tac (*a*) or UPC10 (lane *b*) as described in Fig. 3 legend, and then electrophoresed on 7.5% SDS gels and autoradiographed. Molecular weight markers are indicated on the left.

Luteinizing hormone release from dissociated pituitary cells by dimerization of occupied LHRH receptors

H. Gregory & C. L. Taylor

ICI Pharmaceuticals Division, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

C. R. Hopkins

Department of Medical Cell Biology, University of Liverpool, Liverpool L69 3BX, UK

Studies of luteinizing hormone-releasing hormone (LHRH) have revealed several structural requirements for its biological activity[1]. Shortening of the decapeptide leads to loss of activity; positions 1, 6 and 10 are regarded as important in that they have a high affinity for the hormone receptor, and positions 2 and 3 (His-Trp) are thought to comprise the active centre of the molecule (replacement of His or Trp produces the most effective antagonists). Using a potent agonist analogue of the decapeptide, pGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-NHEt, coupled to ferritin, we have demonstrated previously the binding, aggregation and internalization of bound hormone using dissociated pituitary cells[2]. Similar processes occur for other biologically active peptides, for example, insulin[3] and epidermal growth factor (EGF)[4]. Using a pure antagonist in the simpler LHRH system we now show that the N-terminal portion of the molecule is important for receptor aggregation and that dimerization of occupied LHRH receptors is a necessary early stage in the release of luteinizing hormone.

The octapeptide, Z-Gln-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHEt, is an effective inhibitor of LHRH both *in vitro* and *in vivo*[5]. We prepared the 6-D-Lys analogue of this peptide to allow easy substitution at a central position in the molecule;