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Comparative analysis of HIV-1 recombinant envelope glycoproteins from different culture systems

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Abstract The productivity of stable Chinese hamster ovary cell lines secreting HIV-1 monomeric (IIIB gp120) and oligomeric (UG21 gp140) recombinant envelope glycoproteins was compared in serum-containing (S+), serum-free (S−) and protein-free (P−) culture media. UG21 gp140 expression was greatest in S+ medium, while IIIBgp120 production was lower than gp140 in all three media but highest in S−. UG21 gp140 production was highest in standard 850-cm^2 roller bottle cultures in S+ media, peaking after 14 days of incubation, while expression levels in the three media were 0.5 (S+), 0.4 (S−) and 0.2 (P−) mg/l, from which 90, 80 and 12% of gp140, respectively, could be purified by immunoaffinity chromatography. Purified UG21 gp140 from S+ and S− media possessed biological functionality as evidenced by CD4 and monoclonal antibody (Mab) binding. In contrast, UG21 gp140 from P− medium appears to be misfolded and non-functional. Despite the possession of a different N-linked glycan profile, UG21 gp140 from S− media shows very similar CD4 and Mab binding characteristics to S+ UG21 gp140. The relevance of these findings to HIV vaccine development is discussed.

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Introduction

As the AIDS pandemic continues to spread, there is an urgent requirement for purified, functional HIV-1 recombinant envelope glycoprotein not only as a potential vaccine but also to (a) generate reagents, particularly monoclonal antibodies, and (b) to characterise the immunological responses from vaccines (Moore et al. 2001). Although recombinant HIV-1 envelope glycoprotein has been available for nearly 20 years, it has mostly been derived from North American and European B-clade isolates, whereas the highest levels of HIV-1 prevalence are currently in sub-Saharan Africa and Asia, where non-B-clade strains predominate (UNAIDS 2002). To redress this imbalance, our laboratory has been involved with two European-Union-funded vaccine initiatives, The European Vaccine against AIDS (EVA) and EuroVac, where our role has been to produce a range of monomeric (gp120) and oligomeric (gp140 = gp120 + external domain of gp41) recombinant envelope glycoproteins encompassing clades A, B, C, D, F and O (Jeffs et al. 1999, 2002, 2004). Part of our remit has been to optimise glycoprotein production in a variety of small-scale culture systems to develop purification strategies and to characterise the potential biological functionality of the purified gp120s and gp140s.

This report investigates the growth and productivity of gp120- and gp140-expressing Chinese hamster ovary (CHO) cell lines in a number of different culture systems, and compares and contrasts the biological functionality of the purified glycoproteins. The gp120 line used in this study was derived from the North American B-clade T-cell-line-adapted isolate IIIB (BH10 molecular clone), while the gp140 line was derived from the African D-clade primary isolate 92/UG/021 (92UG021-16 molecular clone). UG21 gp140 is a stable equimolar mix of dimeric

and trimeric envelopes (Jeffs et al. 2004). Our choice of a recombinant CHO cell production system was dictated by the high degree of glycosylation of HIV envelope glycoprotein (Montagnier et al. 1985), which could not be achieved in a bacterial expression system, and the fact that the antennary structures of glycoproteins (including HIV-1 gp120) expressed from CHO cells more closely resemble those produced in human cells than do the high-mannose glycoproteins obtained from the baculovirus/insect cell (or yeast) expression systems (Moore et al. 1990).

There is also a move towards the use of serum-free (S−) medium and a need to eliminate substances of animal origin (particularly serum) in biopharmaceutical production to avoid contamination with viruses and transmissible spongiform encephalopathies (TSEs), improve reproducibility and optimise product recovery (Froud 1999; Mauer 1992; Castle and Robertson 1999). During the adaption process to S− suspension, growth conditions may be vulnerable to outgrowth of undesirable subpopulations of cells that may show low growth rate, poor specific productivity or genetic instability (Zang et al. 1995; Ozturk and Palsson 1991). For all these reasons the growth characteristics of recombinant cell lines as well as the structural and functional integrity of the expressed protein must be closely monitored throughout the development process.

To investigate the effects of S- and protein-free (P−) culture conditions on both the productivity of gp120/140 CHO cell lines and the functionality of gp120/140 purified from these systems, we have compared the productivity of IIIB and UG21 CHO lines initially in medium containing foetal bovine serum (FBS; S+) then during stepwise adaption to S− and P− media. One UG21 line from each media was scaled-up to provide sufficient immunoaffinity purified gp for structural characterisation by CD4 receptor/monoclonal antibody (Mab) binding and N-linked glycan analysis and to compare the productivity of adherent cell and suspension culture systems. In phase 2, UG21 gp140 production in S+ and S− media was compared in a number of medium-scale adherent or suspension cell culture systems: tissue culture (T) flasks, roller bottles (normal and surface-enhanced) and spinner flasks. Again, purified gp140 was characterised by receptor/Mab binding and glycan analysis. From this analysis, conclusions can be drawn as to the optimal culture system for the medium-scale production of functional recombinant envelope glycoprotein.

Materials and methods

Cell lines

Two stable CHO lines were employed in this study. ARP248 secretes recombinant monomeric IIIB (BH10 molecular clone) gp120, while UG21 secretes recombinant oligomeric UG21 gp140 into the medium. gp140 is gp120 plus the external domain of gp41. The gp120/41 cleavage site is retained. Both lines were generated by transfection of CHO-K1 cells with pEE14/tissue plasminogen activator (tPA)/gp120(140) vectors, in which the HIV *env* gene signal sequence is replaced by that of tPA to maximise translocation and secretion (Chapman et al. 1991). tPA is cleaved upon exit from the cell. ARP248 was supplied by the EU Programme EVA/MRC Centralised Facility for AIDS Reagents [CFAR, National Institute for Biological Standards and Control (NIBSC), Herts, UK]. For full details of the UG21 cell line, see Jeffs et al. (2004).

Parent CHO-K1 cell lines were obtained from the European Collection of Cell Cultures (ECACC No. 85051005, Porton Down, UK) and screened for mycoplasma before use and prior to the establishment of master cell banks.

Culture media

Both cell lines use the glutamine synthetase (GS) expression system (Cockett et al. 1991), which has been routinely used for the production of fully functional HIV-1 gp120/140. Full details of growth and culture conditions are given in Jeffs et al. (1996, 2004).

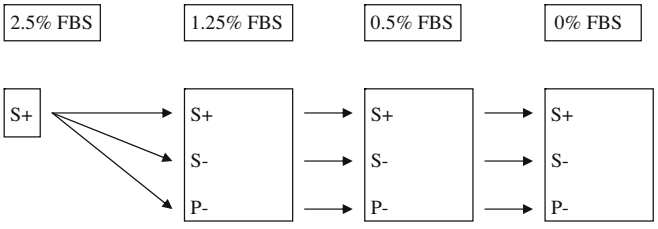
Following initial growth in CB2 medium (Gibco, UK) supplemented with 2.5% dialysed FBS (Gibco-BRL, USA) + 25 (ARP248) or 200 (UG21) μM L-methionine sulfoximine (MSX) (hereafter "S+ media"), both lines were subcultured in either S− or P− media, initially supplemented with 2.5% FBS then adapted stepwise to progressively lower concentrations of FBS (see "Results"). The S− medium employed was Excell 302+, and the P− medium was Excell 325-PF+ (JRH Biosciences, UK), both supplemented with 1X GS supplement (JRH Biosciences).

Cell culture

Phase I

Both cell lines were initially grown in S+ media in T25 flasks. To determine cell-specific production rates (CSPRs), at c. 90% confluence, the medium was replaced with three batches of 5 ml fresh medium for 3×24-h periods, each 24-h medium batch being assayed for gp120/140 (see "gp purification, quantification ELISAs, Mab and CD4 binding assays"). After the third harvest, cells were removed by treatment with trypsin/EDTA at 4°C to minimise damage, briefly centrifuged (400×*g* for 5 min at 4°C), resuspended in 5 ml media and counted. An aliquot of cells was assessed for viability by staining in 0.4% trypan blue, and their gross morphology was assessed by light microscopy. A constant number of cells (7.5×10^4)

were used for each subcloning step, with a minimum of three passages at each concentration of FBS. T25 flasks were used throughout, with each experiment performed in triplicate. The stepwise adaption to S− and P− media is given below:



Once adapted to S+ (0.5% FBS), S− (0% FBS) and P− (0% FBS), the cultures were transferred to:

S+: Cells adherent, scaled-up to 2 × T175 flasks then used to seed 2 l of S+ (0.5% FBS) medium for 1× 10-tray Nunc Cell Factory; cell-conditioned culture supernatant (TCSN) harvested after 7 days.

S−: Cells formed large, non-adherent spheroidal masses; scaled-up to 2 × T175 flasks then transferred to spinner flasks (40 rpm for 3 days, increasing to 60 rpm) in 300 ml S− medium; TCSN harvested after 7 days.

P−: Cells formed small, non-adherent spheroidal masses; scale-up to spinner flasks and harvest TCSN as for S−.

Cell culture

Phase II

UG21 gp140 cell lines adapted to growth in S+ (2.5% FBS-adherent line) and S- (0% FBS-suspension culture) media were cultured in one of four systems:

1. Tissue culture flask (175-cm^2)
2. Standard 850-cm^2 roller bottle
3. "Expanded surface area" 850-cm^2 roller bottle containing silicon rubber matrix ("ImmobaSil"; Ashby Scientific Ltd., Corby, UK)
4. 1 l spinner flask

T175 flasks (5×10^7 cells) of confluent, adherent UG21 cells in S+ (2.5% FBS) medium were resuspended in 50 ml S+ medium per flask to give 1×10^6 cells per millilitre then transferred to culture vessels as follows:

(a) Standard and expanded surface area 850-cm^2 roller bottles; input of 10×10^6 cells in 200 ml S+ medium; TCSN harvested and replaced at 3- to 4-day intervals.
(b) Tissue culture flasks (4×175 cm^2); input of 5×10^6 cells in 50 ml S+ medium per flask; TCSN harvested/replaced at 3- to 4-day intervals.

Three T175 flasks (0.5×10^7 cells by nuclei counts per flask) of aggregated UG21 cells in S− (0% FBS) medium were pooled and resuspended in 500 ml S− medium to give 0.3×10^6 cells per millilitre then transferred to culture vessels as follows:

(a) Cell suspension (200 ml; ca. 6×10^6 cells) transferred to a standard roller bottle (15 revolutions per hour); TCSN harvested/replaced at 3- to 4-day intervals.
(b) Cell suspension (300 ml; ca. 9×10^6 cells) transferred to a spinner flask (40 rpm for days 0–7; 60 rpm thereafter); 200 ml TCSN harvested and replaced with 200 ml fresh medium (dilution of 1:2) at 3- to 4-day intervals.

For cells incubated in roller bottles, TCSN was harvested and replaced as above for up to 25 days, and gp140 production was monitored throughout by quantification enzyme-linked immunosorbent assay (ELISA). Having established the time of peak production (14 days), in a subsequent experiment using identical culture conditions, medium was changed at days 4, 7 and 11, then cells and TCSN were harvested after 14 days. gp140 purified from TCSN with D7324 immunoaffinity chromatography (Jeffs et al. 1996, 2004) was then characterised for CA13/2G12/CD4 binding and N-linked glycan profile. All phase I and II experiments were repeated at least twice.

gp purification, quantification ELISAs, Mab and CD4 binding assays

UG21 gp140 from S+, S− and P− large-scale cultures was immunoaffinity-purified with immobilised D7324 as detailed in Jeffs et al. (1996, 2004).

Two sandwich ELISAs were used to quantify IIIB gp120 and UG21 gp140. For IIIB gp120, gp was captured with the ovine anti-peptide polyclonal sera D7324 (Aalto Bioreagents, Dublin, Ireland) then detected with a CHO-derived $IIIB_{BH10}$ gp120 rabbit antisera (ARP421-CFAR). Standard curves were generated using CHO $IIIB_{BH10}$ gp120 (EVA657-CFAR).

UG21 gp140 was captured by *Galanthus nivalis* lectin (Vector Laboratories, Peterborough, UK) and detected with a rabbit CHO UG21 gp140 antisera. Standard curves were produced using purified CHO UG21 gp140. Full details of both ELISAs are given in Jeffs et al. (1996, 2004).

The antigenic topology of UG21 gp140 purified from all three media was assessed by Mab and soluble CD4 (sCD4) binding using the ELISAs and immunoblots detailed in Jeffs et al. (1996, 2004). Two Mabs were employed: CA13 (ARP3119-CFAR), which recognises a linear epitope in the C1 region of W61D gp120 (Arnold and Kent, unpublished results) and 2G12 (EVA3064-CFAR), which recognises a conformation and carbohydrate-dependent epitope in the C4/V4 regions of IIIB gp120 (Buchacher et al. 1994).

Glycan analysis

Release and labelling of N-glycans

Gp140 N-glycans were released by digestion with peptide/N-glycosidase F (PNGase F; New England Biolabs, Hitchin, UK) and labelled with 4-ABA as described by Yuen et al. (2002). In brief, lyophilised rgp140 (25 μg) was digested with 1,000 U of PNGase F using the reagents supplied by the manufacturer in a 100 μl reaction volume at 37°C for 20–24 h. The N-glycans were collected, purified and their hexose content estimated using orcinol staining (Feizi et al. 1994). Lyophilised oligosaccharides (up to 100 μg) in a 1 ml vial were dissolved in 4-ABA solution [10 μl; 2 M in 7:3 dimethyl sulphoxide (DMSO)/HOAc (v/v)], and tetrabutylammonium cyanoborohydride solution [15 μl; approximately 1.5 M, freshly prepared in 7:3 DMSO/HOAc (v/v)] was added. After heating at 60°C for 4 h, distilled water (200 μl) was added, and excess reagents were removed by extraction five times with ethyl acetate (300 μl). 4-ABA-labelled oligosaccharide products are in the lower aqueous phase.

High-performance liquid chromatography

Diethylaminoethyl (DEAE) anion exchange and normal-phase Amide-80 high-performance liquid chromatography (HPLC) were performed using an inert Gilson binary gradient system (Anachem, Luton, UK) fitted with a model 122 fluorescence detector as described by Yuen et al. (2002). Operation and data acquisition were controlled using the Unipoint software package (Anachem).

Results

Phase I

Growth rate during adaption to S−/P− media

During adaption to S− and P− media (Fig. 1), the growth of UG21 cell lines initially slowed as the FBS concentration was reduced from 2.5 to 1.25% in S+ and S− media [from 4×10^5 to 2×10^5 (S+) and 2.5×10^5 (S−) cells per millilitre] then recovered to 4×10^5 cells per millilitre for S+ medium during reduction of FBS from 1.25 to 0.5%, and to 3.3×10^5 cells per millilitre for S− medium. In contrast, growth in P− medium was enhanced, being nearly double that of growth in S+ medium. Neither of the cell lines survived in FBS-free S+. UG21 cells detached and formed spheroids in FBS-free S− medium, but the IIIB line remained attached. It appeared that expression of monomeric or oligomeric envelope had an effect on cell growth in these small-scale culture systems. There was very little growth crisis of IIIB cells during adaption to S− conditions in all three media, and the cell density was persistently higher in S+ and S− media than that seen with UG21 cells. Growth in P− medium is very similar in both cell lines at 0 and 0.5% FBS, but the IIIB cells showed markedly enhanced growth at 1.25% FBS compared to UG21. Elongation of cells was noted as the FBS level was reduced, but no other gross morphological changes were apparent.

Viability of cells during adaption to S−/P− media

The viability of both lines (Fig. 2) rose as the cells adapted to lower levels of FBS in all three media, although the highest viability was found in P− medium, with progressively lower viabilities in S− and S+. S+ medium could not support the growth of either cell line in the absence of serum. In general, IIIB cells achieved higher viabilities than the UG21 line.

Production of UG21 gp140 and IIIBgp120 in S+/S−/P− media

The data illustrated in Fig. 3 indicates that, although UG21 gp140 product levels dropped as the FBS level was reduced from 2.5 to 1.25%, it was similar in all three media at 1.25% FBS. Production levels were maintained in S+ medium supplemented with 0.5% FBS but decreased by more than 50% in the 0.5% FBS-supplemented S− and P− media. As indicated above, S− and S+ medium did not

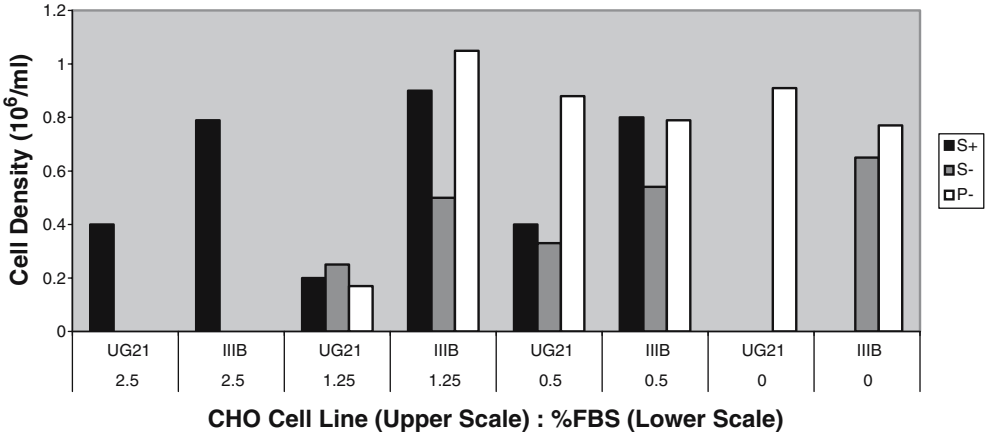


Fig. 1 Growth of UG21 and IIIB CHO cell lines in serum-containing (*S+*), serum-free (*S-*) and protein-free (*P−*) media. [Figs. 1, 2, 3, 4, 5: Each data point is the mean of at least two experiments (error bars omitted for clarity)]

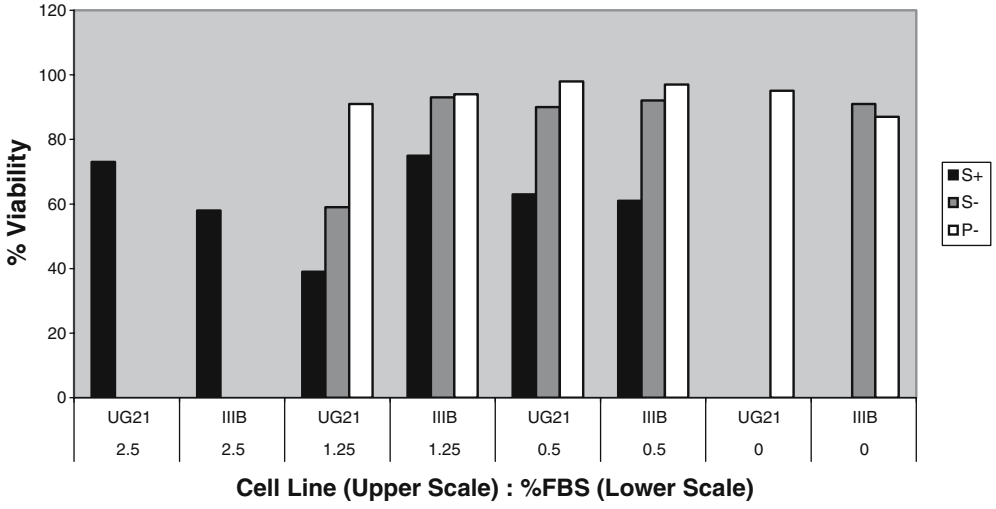


Fig. 2 Percentage viability of cells lines in serum-containing (*S+*), serum-free (*S-*) and protein-free (*P-*) media

support cell growth, and UG21 cells formed spheroids in S− medium and exhibited reduced gp140 production in S− and P− medium. These results also provide further evidence that the production of monomeric and oligomeric envelope in CHO cells differs as follows:

- UG21 expression levels were generally higher than IIIB in S+ medium as FBS supplementation was reduced, and UG21 expression levels did not show such precipitous decline seen with IIIB production.
- Expression in S− media was similar in both cell lines, declining as FBS supplementation was reduced.
- UG21 expression declined in P− medium with adaption, but IIIB expression in the same medium was lower throughout.

CSPRs in S+/S−/P− media

The results given above (Production of UG21 gp140 and IIIBgp120 in S+/S−/P− media) were confirmed by calculation of CSPRs (Fig. 4), which indicated that UG21 gp140 production was generally greater than IIIB gp120 in all growth media except S+ and S− with NO FBS.

- In S+ medium, the UG21 gp140 CSPR fell from 4.0 to 2.6 μg per 10^6 cells per 24 h, as FBS dropped from 2.5 to 0.5%, while the equivalent figures for IIIB were 1.6 falling to 0.5.
- In S− medium, the UG21 CSPR dropped from 3.0 to 1.0 μg per 10^6 cells per 24 h, as FBS was reduced from 1.25 to 0.5% (spheroids in S−), a lower productivity than S+ medium, while the IIIB values fell from 2.0 to 0.2.
- In P− medium, the CSPR values reduced from 0.8 to 0.2 μg per 10^6 cells per 24 h as FBS supplementation was reduced from 1.25 to 0% despite the high viability and growth rate of these cells (Fig. 2).

Thus, within T flasks, the CSPR values suggest that adaption to S− medium was detrimental to gp production, while the use of P− medium does not appear to be appropriate for production of both IIIB gp120 and UG21 gp140 glycoproteins. The highest CSPRs were always obtained with 2.5% FBS-containing media.

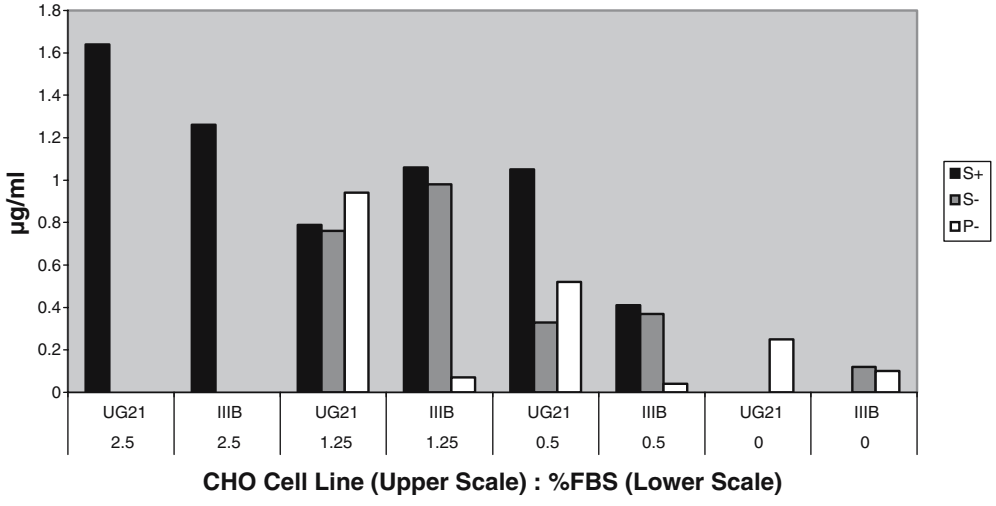


Fig. 3 Expression of UG21 gp140 in serum-containing (*S+*), serum-free (*S-*) and protein-free (*P-*) media

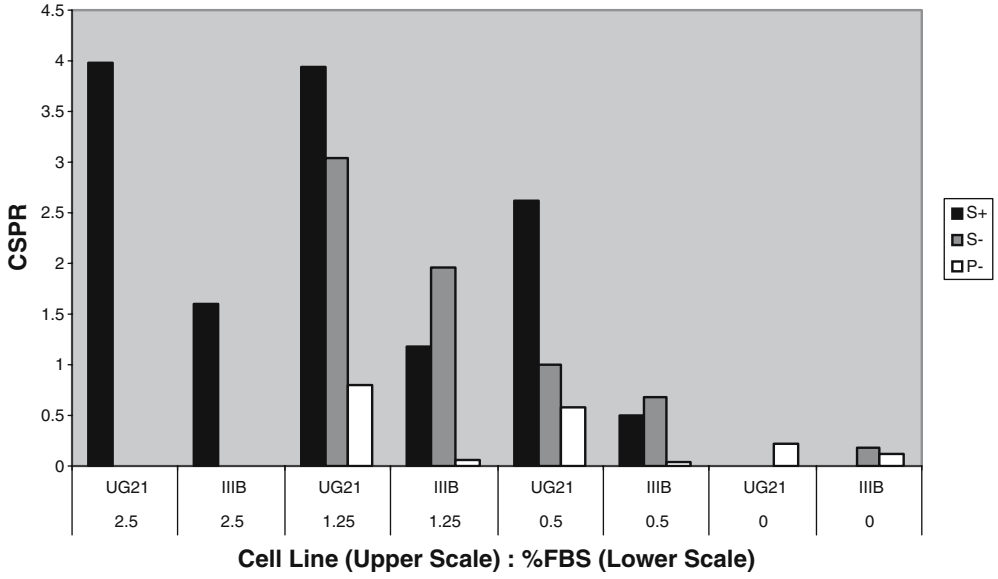


Fig. 4 CSPR (micrograms per 10^6 cells per 24 h) in serum-containing (*S+*), serum-free (*S-*) and protein-free (*P−*) media

Production of UG21 gp140 in larger-scale culture systems

On scaling-up from T flasks to a static, adherent culture system (Cell Factory) for S+/0.5% FBS, or a stirred, suspension culture system (spinner flasks) for S− or P−/0% FBS, yields of UG21 gp140 from the S+ and S− media were similar at 500 and 400 μg/l, respectively. However, expression levels in P− medium were, as seen in T flasks, much reduced at 200 μg/l (Table 1). Ninety, 80 and 10% of gp140 could be immunoaffinity-purified from S+, S− and P− TCSN, respectively. All three purified UG21 gp140s retained their normal oligomeric structure (Jeffs et al. 2004) as determined by non-reducing polyacrylamide gel electrophoresis (data not shown).

Characterisation of purified UG21 gp140: antibody and sCD4 binding

The sCD4 and Mab binding characteristics and the N-linked glycan profile of each purified gp140 are given in Table 1. The results indicate that, although the glycan profile of UG21 gp140 was altered by growth in S− medium, it remained correctly folded as evidenced by its ability to bind sCD4 and the Mabs CA13 and 2G12. In contrast, P− UG21 gp140 was only weakly recognised by CA13 and did not bind sCD4 or 2G12, suggesting it was misfolded. Insufficient P− UG21 gp140 was available for glycan analysis. Taken with the data on glycoprotein production, this strongly suggests that gp140 purified from P− TCSN is of insufficient quantity or quality to be considered for HIV vaccine production.

Characterisation of purified UG21 gp140: N-linked glycan analysis

Purified UG21 gp140 derived from S+ cultures of the UG21 cell line contained a full range of N-glycans from neutral (approximately 70% of total) to acidic (approximately 30% of total) (Table 1). Neutral N-glycans were mainly of the high-mannose type eluting at the same positions as standards Man 5, Man 6, Man 7 and Man 8 in the ratio 2:1:2:0.5. The major component of the acidic N-glycans was monosialylated biantennary with fucose, A1F (approximately 50% acidic). The remainder was disialylated biantennary structure + fucose (approximately 25% acidic) and tri- and tetrasialyated N-glycans with or without fucose and/or N-acetyllactosamine extension.

S− medium gp140 N-linked glycans were mainly composed of Man 7 (70% total) and A1F (20% total).

Table 1 Production and characterisation of purified UG21 gp140 from larger-scale culture (phase I)

Medium	Yield (μg/l)	Purified[a] [μg, (%)]	Assay			
			sCD4 binding	CA13 binding	2G12 binding	Glycan profile[b]
S+	500	450 (90)	+++	+++	+++	70% N:30% A
S−	400	320 (80)	+++	+++	+++	100% N
P−	200	20 (10)	−	+	−	ND

+++ Strong binding (>×10 background), − no specific binding discernable, + weak binding (×1–2.5 background)
N neutral carbohydrate residues, *A* acidic residues *ND* not determined (too little and misfolded)
[a]Purified by Aalto D7324 immunoaffinity chromatography (in micrograms and as percentage of amount in TCSN prior to purification)
[b]N-linked glycan profile is ratio of neutral (N) to acid residues (A)

Table 2 Characterisation of UG21 gp140 from different culture systems at day-14 harvest (phase II)

System	Media	Yield μg/800 ml	Total cells × 10^6	CSPR[a]	Glycans[b]	Binding		
						sCD4[c]	CA13	2G12
Standard roller	S+	852	108	2.0	70:30	+++	Y	Y
Enhanced roller	S+	556	100	1.4	70:30	+++	Y	Y
T175 flask	S+	686	34	5.0	70:30	+++	Y	Y
Standard roller	S−	58	3	4.8	90:10	−	Y	N
Spinner flask	S−	480	318	0.4	80:20	+++	Y	Y

Y Binds in immunoblot, *N* does not bind
[a]CSPR in micrograms per 24 h per 10^6 viable cells
[b]See Table 1 for legends
[c]See Table 1 for legends

The remaining 10% were Man 5 (5% total) and other high-mannose structures, but no higher acidic structures were detected.

Thus, growth in S− medium significantly altered the N-linked glycan profile. This did not seem to affect the three-dimensional structure of gp140 (Tables 1 and 2), but further work will be required to determine if immunogenicity was affected by such variation.

Phase II

Values for UG21 gp140 production and cumulative yield in five different medium-scale culture systems are given in Figs. 5 and 6. Taking each system in turn:

S+/standard roller bottle: gp140 production peaked after 14 days (4.3 μg/ml) then declined as cells progressively detached. Cumulative yield at day 14=2,192 μg (800 ml).

S+ enhanced-surface-area roller bottle: gp140 production again peaked at 14 days (2.8 μg/ml), followed by a slow decline in production to 22 days then extensive cell detachment and death after 25 days. Cumulative yield at day 14=1,756 μg (800 ml).

S+ T175 flask: Rapid build-up in production after only 7 days of incubation, then relatively stable production throughout [peak (3.8 μg/ml) at 22 days]. A constant monolayer of cells was retained throughout, with no extensive cell detachment. Cumulative yield at day 14=2,198 μg (800 ml).

S− spinner flask: Generally low production, peaking at 14 days (2.4 μg/ml), with subsequent and rapid loss of cell viability. Cumulative yield at day 14=1,296 μg (800 ml).

S− standard roller bottle: Cells had difficulty in attaching and growing in a monolayer in this system and tended to detach and form small spheroids. Production was consequently very low, peaking at 10 days (0.5 μg/ml). Cumulative yield at day 14=192 μg (800 ml).

gp 140 production was in the order (highest first) S+ standard roller bottle = S+ T175 flask > S+ enhanced-surface-area roller bottle > S− spinner flask > S− standard roller bottle. Overall productivity was thus higher in S+ attached cell culture than S− suspension systems.

Having established the time of peak production (14 days), a repeat experiment was performed to compare CSPR values and antigenic topology of purified protein from all five systems. The results are given in Table 2, and

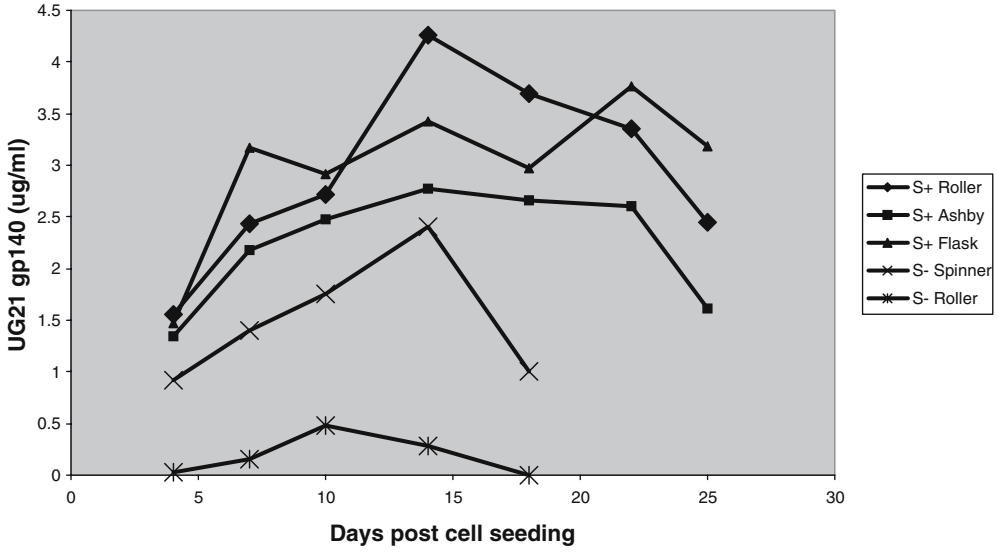


Fig. 5 Production of UG21 gp140 in different culture systems

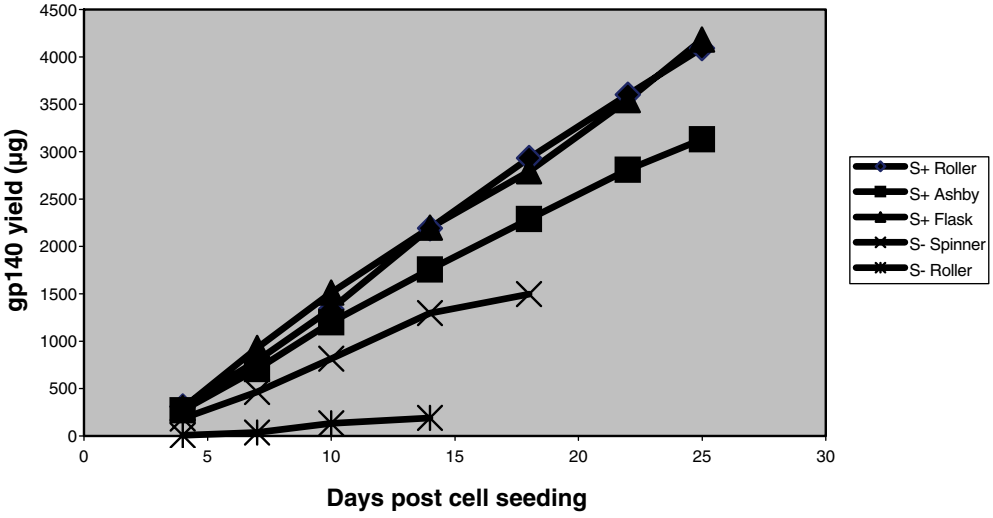


Fig. 6 Cumulative yield of UG21 gp140 in various culture systems

the results indicate that the highest protein yields were again provided by attached, S+ systems. However, cell density varied widely, from very low in S− standard roller bottles, intermediate in the three S+, attached cell systems and highest in the S− suspension culture in the spinner flask. Nevertheless, despite this high cell density in the latter system, protein expression was low, resulting in the lowest CSPR. In fact, although cell density was very low, the few cells in the S− standard roller bottle were high expressors, resulting in the second highest CSPR. Unfortunately, gp140 purified from this system is likely to be misfolded and non-functional (Table 2), and it cannot be recommended for use. All purified gp140s (except that from S− standard roller bottles) had the binding characteristics of glycoproteins with native folding irrespective of glycan profile.

Discussion

There is good evidence for both the differential production of oligomeric and monomeric HIV-1 envelope and cell growth from recombinant CHO cells in small-scale (T flask) adherent culture systems. The results from adaptation of cell lines to S− culture ("Results") of this study indicate that UG21 cell lines undergo a growth crisis during this process, with cell growth dropping as the concentration of FBS falls from 2.5 to 1.25%, then recovering in media designed for S− growth as serum levels used are reduced. Decline in growth performance due to a crisis in the normal cell cycle progression following serum withdrawal is well established (Zang et al. 1995; Griffiths and Racher 1994; Sinacore et al. 2000). However, IIIB cells appeared to be less affected, possibly surviving due to the formation of large aggregates that may enhance survival through cell–cell/matrix contacts. CSPR values for both IIIB gp120 and UG21 gp140 production showed a marked decline in S− and particularly P− medium when compared to media supplemented with 2.5% FBS. In general, there was higher expression of UG21 gp140 in S+ and S− media, but these same media supported lower cell density than IIIB cells, leading to greater overall CSPR values. In contrast, IIIB gp120 expression in S+ and S− is lower than that of UG21 gp140, and, although IIIB cell densities are generally higher than those of UG21 cells, overall CSPR scores were lower. Both lines proliferated in P− medium with high viabilities, exceeding that seen with standard FBS-containing medium. However, protein expression in P− medium was severely inhibited. The authors are not aware of any similar studies that consider the comparative expression and cell growth of recombinant mammalian cell lines producing monomeric or oligomeric HIV glycoprotein in a variety of culture vessel formats as alternatives for scale-up.

A number of studies have compared the growth and production of recombinant proteins in S− or P− medium to that in S+ medium in attached and suspension cultures. The outcome varies widely with the cell line used, protein expressed and culture medium utilised (Goochee and Monica 1990). In general, adaption of recombinant CHO cells to suspension culture in S− medium led to higher cell growth and enhanced production of a number of recombinant proteins, including human growth hormone (Hata et al. 1992; Haldankar et al. 1999), human endotoxin receptor CD14 (Schutt et al. 1997), human tissue kallikrein (Watson et al. 1994) and human chorionic gonadotropin (hCGT) (Battista et al. 1994). A rCHO line previously adapted to growth in S− medium showed very high growth and expression of urokinase plasminogen activator (uPa) and a humanised immune globulin G (IgG) kappa light chain when adapted to P− medium. Up to 74 mg/l of uPa and 118 mg/l of IgG were obtained in a 2.5-l closed-loop bioreactor (Zang et al. 1995). A very thorough study by Battista et al. (1994) examined the growth of rCHO cell lines expressing hCGT in a variety of both suspension and attached culture systems. Once adapted to S− suspension culture, expression of hCGT was enhanced compared to S+ medium and readily adapted to scale-up in both 5-l stirred tank and 30-l airlift bioreactors. Specific productivities of up to 2.7 µg per 10^6 cell per 24 h in the stirred tank and 2.6 µg per 10^6 cell per 24 h in the airlift bioreactor were obtained. Adherent lines were also adapted to S− conditions and showed enhanced productivity in both T flasks

and roller bottles over S+ media. When glass microcarrier beads were employed as the culture system, rCGT production was seven times higher than that obtained with roller bottles. A similar study looking at the expression of a human interleukin(IL)-2 variant glycoprotein (IL-Mu6) in BHK-21 cells compared yields from suspension and adherent (microcarrier beads) cultures in the presence and absence of 2% FBS using a 2.5-l bioreactor (Gawlitzek et al. 1995). In both systems, IL-Mu6 yield doubled in the absence of FBS and reached its highest productivity of 5.5 mg from 2.3 l with microcarrier beads in the absence of serum.

In our hands, both the yield and CSPR for UG21 gp140 (14 days post-seeding) dropped in the absence of serum, from a yield of 2,192 μg in 800 ml TCSN (CSPR= 2.0 μg/10^6 cells per 24 h) in S+ medium (S+/2.5% FBS/ standard roller bottle) with adherent cells to 1,296 μg/ 800 ml TCSN (CSPR=0.4 μg per 10^6 cells per 24 h) in S− medium (S−/0%FBS/spinner flask) with cells in suspension culture. Although these figures cannot be directly compared to the studies described above, which employed large-scale bioreactors, they do tend to indicate that optimal yields of this complex, oligomeric glycoprotein, are obtained from adherent cells in serum-supplemented medium.

Of equal concern to productivity, however, are the effects that the choice of culture system (cell + media + culture system) can have on the therapeutic effectiveness of a recombinant glycoprotein. A critical factor is the potential for changes in glycosylated product that may occur during the culture process. The results of this study suggest that adaption to S− medium is accompanied by a change in the ratio between neutral and sialyated (acidic) oligosaccharides, with a decline in the latter structures, although this does not appear to affect the three-dimensional structure of gp140 from either S+ or S- media. Geisse et al. (1996) saw no differences in the ability of human leukaemia inhibitory factor derived from five different cell lines to induce the proliferation of DA-1 cells. In contrast, the ADCC activity of the humanised antibody CAMPATH-1H was directly related to the glycosylation profile conferred upon it by the choice of cell line. CAMPATH-1H from YO myeloma lines was consistently more active than those derived from NSO myeloma or CHO cells. However, no differences were noted in the ability or affinity of CAMPATH-1H from all three lines to bind the leucocyte receptor CD52 or in a monocyte killing assay (Lifely et al. 1995). A variety of other studies have noted major differences in the sialic acid content and degree of galactosylation of a murine Mab grown in ascites, S− or S+ medium (Patel et al. 1992), and major differences in the degree of fucosylation, sialyation and antennarity of the N-linked glycans of both IL-Mu6 and human tissue kallikrein grown in the presence or absence of serum in suspension or microcarrier systems (Gawlitzek et al. 1995; Watson et al. 1994). Although such changes appear to be related to productivity of the recombinant glycoprotein, the affects on therapeutic activity remain to be determined. In general, our study showed similar broad changes, with little change apparent in the biological functionality of purified UG21 gp140-derived recombinant CHO lines adapted to S+ or S− medium. However, gp140 from P− medium was found to have characteristics of misfolded and non-functional glycoprotein.

The envelope glycoproteins of human viruses are synthesised and glycosylated essentially by the same mechanisms as human cell glycoproteins (Datema et al. 1987). Thus, the viral protein (including HIV-1 and HIV-2) acquires a specific pattern of high-mannose, complex-type and hybrid-type oligosaccharide structures depending on the host cell employed (Geyer et al. 1988; Biller et al. 1998; Taverna et al. 1999). Gp120s from primary viral isolates (obtained from peripheral blood mononuclear cells) of HIV-1 contain between 23 and 26 N-linked glycosylation sites, about half of which are high-mannose structures and the remainder hybrid/complex types. The high-mannose glycans tend to be located in clusters within the gp120 domains that are conserved among HIV-1 isolates, whereas the complex/hybrid structures are more likely to be found on the hypervariable loops (Sanders et al. 2002; Scanlan et al. 2002). CHO gp120 is similar to native envelope, being composed of high-mannose, complex and hybrid carbohydrates (Mizuochi et al. 1988, 1990; Zhu et al. 2000), with a ratio of around 13–15 high-mannose to 10–11 complex/ hybrid structures for the 4 B-clade isolates analysed to date. This represents a ratio of neutral (high mannose)/acidic (complex/hybrid) of between 57 and 65% neutral/43 and 35% acidic, which is close to the 70:30% figure obtained with S+ medium in this study for a D-clade isolate. In contrast, gp120 derived from the baculovirus/insect cell expression system is entirely composed of high-mannose structures (Yeh et al. 1993). Gp120 from bacteria is not glycosylated (Morikawa et al. 1990), while yeast expression systems are capable of catalysing disulphide cross-linkage of the nascent polypeptide and simple N-linked glycosylation (Steimer and Haigwood 1991).

There is a strong correlation between the strength of antibody/oligomeric envelope glycoprotein interaction and virus neutralization (reviewed by Sattentau et al. 1999). Thus, alterations in this interaction due to changes in glycosylation are likely to impact upon the efficacy of a recombinant envelope-based immunogen in generating neutralizing antisera. An early study comparing CHO and baculovirus-derived IIIB gp120 indicated that CD4 and Mab binding ability of CHO gp120 was stronger than that of baculovirus gp120 and was considered to more closely resemble that of the native envelope complex (Moore et al. 1990). A more relevant approach derives from an examination of the respective abilities of recombinant envelope proteins derived from different expression systems to induce neutralizing antibody responses in non-human primates or human vaccine trials, or, alternatively, the relative ability of simian immunodeficiency virus (SIV)/ HIV post-infection sera to bind differentially glycosylated envelope proteins. An analysis of the humoral immunogenicity of baculovirus-derived gp120/gp160 candidate human vaccines (VaxSyn) indicated that such preparations were poor antigens in that none of the Mabs produced neutralized the HIV-1 strains tested, and some even appeared to enhance infection (Bristow et al. 1994). One

Mab, B/33, which maps to the V2 domain, was shown to have no neutralizing activity, whereas four similar Mabs mapping to the same V2 peptides but produced using CHO-derived $IIIB_{BH10}$ gp120 all showed potent neutralizing ability (McKeating et al. 1993). Similar poor responses were also seen in human volunteers vaccinated with VaxSyn gp160 (Viscidi et al. 1990; Redfield et al. 1991) whereas, in contrast, CHO-derived $IIIB_{BH10}$ gp120 (Genentech) induced antibodies that recognised both the parent immunogen and baculovirus-derived gp120, bound strongly to V3 loop peptides, blocked CD4 interactions, inhibited syncytium formation and neutralized both heterologous and homologous viruses (Schwartz et al. 1993). Similar results were noted when the structure and antigenicity of recombinant influenza haemagglutinin produced using CHO or baculovirus expression systems were compared (Kuroda et al. 1990, 1991). Sera from macaques vaccinated with baculovirus-derived SIV gp140 also failed to compete with the binding of conformational Mabs, whereas SIV CHO gp140/160 antisera blocked such interactions (Silvera et al. 1994).

Although other factors such as incomplete processing of gp160 to gp120/41 may explain such results, it was most likely that the differential glycosylation of insect-cell-/baculovirus- and mammalian-cell-derived recombinant glycoproteins had a major effect on vaccine immunogenicity, and this must be taken into account when choosing an expression system to produce potential vaccines in the future. Further studies are planned to investigate the effects of differential N-linked glycosylation using gp120/140 derived from CHO cells cultured under S− suspension culture and S+ adherent culture, as well as gp120/140 produced using the baculovirus/insect cell expression system, on immunogenicity.

From our data it is clear that both the culture system and medium can produce marked changes in productivity, structure and quality (i.e. ligand binding and N-linked glycan profile) of HIV-1 gp120/140 expressed in recombinant CHO cells. We have shown that a number of simple assays can be used to measure these parameters. The results presented in this study indicate that correctly folded, fully functional UG21 gp140 can be obtained from a number of different culture systems, and the selection of which to employ is ultimately determined by final glycoprotein yield and ease of use. Although the greatest CSPR is provided by adherent cells in T175 flasks in the presence of 2.5% FBS, this system requires the use of many flasks and is liable to contamination. The authors do not recommend it for the medium-scale production of gp140. Suspension cultures in spinner flasks grow to a high cell density in the absence of serum, but productivity is relatively low, and the majority of cells are lost during the first 14 days post-seeding, leading to reduced total cumulative yield. The most robust system appears to be conferred by attached cell lines in standard roller bottles in the presence of 2.5% FBS. Although the CSPR is half that seen with the T175 flasks, this high-volume system leads to a greater cumulative yield and can be continually harvested over 22 days. The use of enhanced-surface-area roller bottles did not confer in our hands any additional advantage over standard roller bottles because higher cell densities do not seem to have been established, which may have been related to the batch of vessels supplied. Cells adapted to S− media grow very poorly in standard roller bottles. Thus, the system of choice for optimal yield of correctly folded UG21 gp140 is S+/2.5% FBS in standard roller bottles.

To conclude, this study has shown the importance of evaluating the desired quality of recombinant HIV envelope glycoprotein expressed in mammalian cells in a range of alternative media and culture vessel systems before progressing to the development of a large-scale production system. Furthermore, it has also demonstrated the vital importance of using in vitro techniques to evaluate the potential functionality of glycoproteins at an early stage in process development.

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