U.S. Letters Patent No. 4,399,216 and European Patent Applications 117058, 117059 and 117060, all published August 29, 1984.]

CHO DHFR⁻ cells (DuX-B11) CHO K1 cells, Urlaub,
5 et al., Proc. Nat. Acad. Sci. (U.S.A.), Vol. 77, 4461 (1980) lack the enzyme dihydrofolate reductase (DHFR) due to mutations in the structural genes and therefore require the presence of glycine, hypoxanthine, and thymidine in the culture media. Plasmids pDSVL-MkE (Example
10 6) or pDSVL-gHuEPO (Example 7B) were transfected along with carrier DNA into CHO DHFR⁻ cells growing in media containing hypoxanthine, thymidine, and glycine in 60 mm culture plates. Plasmid pSVgHuEPO (Example 7A) was mixed with the plasmid pMG2 containing a mouse dihydrofolate
15 reductase gene cloned into the bacterial plasmid vector pBR322 (per Gasser, et al., supra.) The plasmid mixture and carrier DNA was transfected into CHO DHFR⁻ cells. (Cells which acquire one plasmid will generally also acquire a second plasmid). After three days, the cells
20 were dispersed by trypsinization into several 100 mm culture plates in media lacking hypoxanthine and thymidine. Only those cells which have been stably transformed with the DHFR gene, and thereby the EPO gene, survive in this media. After 7-21 days, colonies of sur-
25 viving cells became apparent. These transformant colonies, after dispersion by trypsinization can be continuously propagated in media lacking hypoxanthine and thymidine, creating new cell strains (e.g., CHO pDSVL-MkEPO, CHO pSVgHuEPO, CHO-pDSVL-gHuEPO).

30 Culture fluids from the above cell strains were tested in the RIA for the presence of recombinant monkey or human EPO. Media for strain CHO pDSVL-MkEPO contained EPO with immunological properties like that obtained from COS-1 cells transfected with plasmid pDSVL-MkEPO. A
35 representative 65 hour culture fluid contained monkey EPO at 0.60 U/ml.

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Culture fluids from CHO pSVgHuEPO and CHO pDSVL-gHuEPO contained recombinant human EPO with immunological properties like that obtained with COS-1 cells transfected with plasmid pSVgHuEPO or pDSVL-gHuEPO. A
5 representative 3 day culture fluid from CHO pSVgHuEPO contained 2.99 U/ml of human EPO and a 5.5 day sample from CHO pDSVL-gHuEPO had 18.2 U/ml of human EPO as measured by the RIA.

The quantity of EPO produced by the cell strains
10 described above can be increased by gene amplification giving new cell strains of greater productivity. The enzyme dihydrofolate reductase (DHFR) which is the product coded for by the DHFR gene can be inhibited by the drug methotrexate (MTX). More specifically, cells propa-
15 gated in media lacking hypoxanthine and thymidine are inhibited or killed by MTX. Under the appropriate conditions, (e.g., minimal concentrations of MTX) cells resistant to and able to grow in MTX can be obtained. These cells are found to be resistent to MTX due to an
20 amplification of the number of their DHFR genes, resulting in increased production of DHFR enzyme. The surviving cells can, in turn, be treated with increasing concentrations of MTX, resulting in cell strains containing greater numbers of DHFR genes. "Passenger genes"
25 (e.g., EPO) carried on the expression vector along with the DHFR gene or transformed with the DHFR gene are frequently found also to be increased in their gene copy number.

As examples of practice of this amplification
30 system, cell strain CHO pDSVL-MkE was subjected to increasing MTX concentrations (0 nM, 30 nM and 100 nM). Representative 65-hour culture media samples from each amplification step were assayed by RIA and determined to contain 0.60, 2.45 and 6.10 U/ml, respectively. Cell
35 strain CHO pDSVL-gHuEPO was subjected to a series of increasing MTX concentrations of 30 nM, 50 nM, 100 nM,

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200 nM, 1 µM, and 5 µM MTX. A representative 3-day culture media sample from the 100 nM MTX step contained human EPO at 3089 ± 129 u/ml as judged by RIA. Representative 48 hour cultural medium samples from the
5 100 nM and 1 µM MTX steps contained, respectively, human EPO at 466 and 1352 U/ml as judged by RIA (average of triplicate assays). In these procedures, 1 x 10^6 cells were plated in 5 ml of media in 60 mm culture dishes. Twenty-four hours later the media were removed and
10 replaced with 5 ml of serum-free media (high glucose DMEM supplemented with 0.1 mM non-essential amino acids and L-glutamine). EPO was allowed to accumulate for 48 hours in the serum-free media. The media was collected for RIA assay and the cells were trypsinized and counted. The
15 average RIA values of 467 U/ml and 1352 U/ml for cells grown at 100 nM and 1 µM MTX, respectively, provided actual yields of 2335 U/plate and 6750 U/plate. The average cell numbers per plate were 1.94 x 10^6 and 3.12 x 10^6 cells, respectively. The effective production
20 rates for these culture conditions were thus 1264 and 2167 U/10^6 cells/48 hours.

The cells in the cultures described immediately above are a genetically heterogeneous population. Standard screening procedures are being employed in an
25 attempt to isolate genetically hemogeneous clones with the highest production capacity. See, Section A, Part 2, of "Points to Consider in the Characterization of Cell Lines Used to Produce Biologics", June 1, 1984, Office of Biologics Research Review, Center for Drugs and
30 Biologics, U.S. Food and Drug Administration.

The productivity of the EPO producing CHO cell lines described above can be improved by appropriate cell culture techniques. The propagation of mammalian cells in culture generally requires the presence of serum in
35 the growth media. A method for production of erythropoietin from CHO cells in media that does not contain

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serum greatly facilitates the purification of erythropoietin from the culture medium. The method described below is capable of economically producing erythropoietin in serum-free media in large quantities sufficient for
5 production.

Strain CHO pDSVL-gHuEPO cells, grown in standard cell culture conditions, are used to seed spinner cell culture flasks. The cells are propagated as a suspension cell line in the spinner cell culture flask in media con-
10 sisting of a 50-50 mixture of high glucose DMEM and Ham's F12 supplemented with 5% fetal calf serum, L-glutamine, Penicillin and Streptomycin, 0.05 mM non-essential amino acids and the appropriate concentration of methotrexate. Suspension cell culture allows the EPO-produc-
15 ing CHO cells to be expanded easily to large volumes. CHO cells, grown in suspension, are used to seed roller bottles at an initial seeding density of 1.5 x 10^7 viable cells per 850 cm^2 roller bottle in 200 ml of media. The cells are allowed to grow to confluency as an adherent
20 cell line over a three-day period. The media used for this phase of the growth is the same as used for growth in suspension. At the end of the three-day growth period, the serum containing media is removed and replaced with 100 ml of serum-free media; 50-50 mixture
25 of high glucose DMEM and Ham's F12 supplemented with 0.05 mM non-essential amino acids and L-glutamine. The roller bottles are returned to the roller bottle incubator for a period of 1-3 hours and the media again is removed and replaced with 100 ml of fresh serum-free
30 media. The 1-3 hour incubation of the serum-free media reduces the concentration of contaminating serum proteins. The roller bottles are returned to the incubator for seven days during which erythropoietin accumulates in the serum-free culture media. At the end of the seven-
35 day production phase, the conditioned media is removed and replaced with fresh serum-free medium for a second

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production cycle. As an example of the practice of this production system, a representative seven-day, serum-free media sample contained human erythropoietin at 3892±409 U/ml as judged by the RIA. Based on an estimated cell density of 0.9 to 1.8 x 10^5 cells/cm^2, each 850 cm^2 roller bottle contained from 0.75 to 1.5 x 10^8 cells and thus the rate of production of EPO in the 7-day, 100 ml culture was 750 to 1470 U/10^6 cells/48 hours.

Culture fluids from cell strain CHO pDSVL-MkEPO carried in 10 nM MTX were subjected to RIA *in vitro* and *in vivo* EPO activity assays. The conditioned media sample contained 41.2 ± 1.4 U/ml of MkEPO as measured by the RIA, 41.2 ± 0.064 U/ml as measured by the *in vitro* biological activity assay and 42.5 ± 5 U/ml as measured by the *in vivo* biological activity assay. Amino acid sequencing of polypeptide products revealed the presence of EPO products, a principle species having 3 residues of the "leader" sequence adjacent the putative amino terminal alanine. Whether this is the result of incorrect membrane processing of the polypeptide in CHO cells or reflects a difference in structure of the amino terminus of monkey EPO *vis-a-vis* human EPO, is presently unknown.

Culture fluids from cell strain CHO pDSVL-gHuEPO were subjected to the three assays. A 5.5 day sample contained recombinant human EPO in the media at a level of 18.2 U/ml by RIA assay, 15.8 ± 4.6 U/ml by *in vitro* assay and 16.8 ± 3.0 U/ml by *in vivo* assay.

Culture fluid from CHO pDSVL-gHuEPO cells prepared amplified by stepwise 100 nM MTX were subjected to the three assays. A 3.0 day sample contained recombinant human EPO at a level of 3089 ± 129 U/ml by RIA, 2589 ± 71.5 U/ml by *in vitro* assay, and 2040 ± 160 U/ml by *in vivo* assay. Amino acid sequencing of this product reveals an amino terminal corresponding to that designated in ~~Table VI~~ Figure 6.

Cell conditioned media from CHO cells transfected with plasmid pDSVL-MkE in 10 nM MTX were pooled,

and the MTX dialyzed out over several days, resulting in media with an EPO activity of 221 ± 5.1 U/ml (EPO-CCM). To determine the *in vivo* effect of the EPO-CCM upon hematocrit levels in normal Balb/C mice, the following experiment was conducted. Cell conditioned media from untransfected CHO cells (CCM) and EPO-CCM were adjusted with PBS. CCM was used for the control group (3 mice) and two dose levels of EPO-CCM -- 4 units per injection and 44 units per injection -- were employed for the experimental groups (2 mice/group). Over the course of 5 weeks, the seven mice were injected intraperitoneally, 3 times per week. After the eighth injection, average hematocrit values for the control group were determined to be 50.4%; for the 4U group, 55.1%; and, for the 44U group, 67.9%.

Mammalian cell expression products may be readily recovered in substantially purified form from culture media using HPLC (C_4) employing an ethanol gradient, preferably at pH7.

A preliminary attempt was made to characterize recombinant glycoprotein products from conditioned medium of COS-1 and CHO cell expression of the human EPO gene in comparison to human urinary EPO isolates using both Western blot analysis and SDS-PAGE. These studies indicated that the CHO-produced EPO material had a somewhat higher molecular weight than the COS-1 expression product which, in turn, was slightly larger than the pooled source human urinary extract. All products were somewhat heterogeneous. Neuraminidase enzyme treatment to remove sialic acid resulted in COS-1 and CHO recombinent products of approximately equal molecular weight which were both nonetheless larger than the resulting asialo human urinary extract. Endoglycosidase F enzyme (EC 3.2.1) treatment of the recombinant CHO product and the urinary extract product (to totally remove carbohydrate from

both) resulted in substantially homogeneous products having essentially identical molecular weight characteristics.

Purified human urinary EPO and a recombinant, 5 CHO cell-produced, EPO according to the invention were subjected to carbohydrate analysis according to the procedure of Ledeen, et al. Methods in Enzymology, 83(Part D), 139-191 (1982) as modified through use of the hydrolysis procedures of Nesser, et al., Anal.Biochem., 10 142, 58-67 (1984). Experimentally determined carbohydrate constitution values (expressed as molar ratios of carbohydrate in the product) for the urinary isolate were as follows: Hexoses, 1.73; N-acetylglucosamine, 1; N-acetylneuraminic acid, 0.93; Fucose, 0; and N-acetyl- 15 galactosamine, 0. Corresponding values for the recombinant product (derived from CHO pDSVL-gHuEPO 3-day culture media at 100 nM MTX) were as follows: Hexoses, 15.09; N-acetylglucosamine, 1; N-acetylneuraminic acid, 0.998; Fucose, 0; and N-acetylgalactosamine, 0. These 20 findings are consistent with the Western blot and SDS-PAGE analysis described above.

Glycoprotein products provided by the present invention are thus comprehensive of products having a primary structural conformation sufficiently duplicative 25 of that of a naturally-occurring erythropoietin to allow possession of one or more of the biological properties thereof and having an average carbohydrate composition which differs from that of naturally-occurring erythropoietin.

30 EXAMPLE 11

The present example relates to the total manufacture by assembly of nucleotide bases of two structural genes encoding the human species EPO sequence of ~~Table VI~~ Figure 6 35 and incorporating, respectively "preferred" codons for expression in E.coli and yeast (S.cerevisiae) cells.

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Also described is the construction of genes encoding analogs of human EPO. Briefly stated, the protocol employed was generally as set out in the previously noted disclosure of Al-n, et al. (WO 83/04053). The genes were
5 designed for initial assembly of component oligonucleotides into multiple duplexes which, in turn, were assembled into three discrete sections. These sections were designed for ready amplification and, upon removal from the amplification system, could be assembled sequentially
10 or through a multiple fragment ligation in a suitable expression vector.

~~Tables VIII through XIV below~~ Figures 10 through 15 and 7 illustrate the design and assembly of a manufactured gene encoding a human EPO translation product lacking any leader or pre-
15 sequence but including an initial methionine residue at position -1. Moreoever, the gene incorporated in substantial part E.coli preference codons and the construction was therefore referred to as the "ECEPO" gene.

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TABLE VIII

ECEPO SECTION 1 OLIGONUCLEOTIDES

No.	Sequence
1.	AATTCTAGAAACCATGAGGGTAATAAAATA
2.	CCATTATTTTATTACCCTCATGGTTTCTAG
3.	ATGGCTCCGCCGCGTCTGATCTGCGAC
4.	CTCGAGTCGCAGATCAGACGCGGCGGAG
5.	TCGAGAGTTCTGGAACGTTACCTGCTG
6.	CTTCCAGCAGGTAACGTTCCAGAACT
7.	GAAGCTAAAGAAGCTGAAAACATC
8.	GTGGTGATGTTTTCAGCTTCTTTAG
9.	ACCACTGGTTGTGCTGAACACTGTTC
10.	CAAAGAACAGTGTTCAGCACAACCA
11.	TTTGAACGAAAACATTACGGTACCG
12.	GATCCGGTACCGTAATGTTTTCGTT

TABLE IX

ECEPO SECTION 1

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      XbaI
EcoRI                 1                          3
AATTCTAG  AAACCATGAG GGTAATAAAA TAATGGCTCC GCCGCGTCTG
    GATC  TTTGGTACTC CCATTATTTT ATTACCGAGG CGGCGCAGAC
                      2                               4

                  5
ATCTGCGACT CGAGAGTTCT GGAACGTTAC CTGCTGGAAG CTAAAGAAGC
TAGACGCTGA GCTCTCAAGA CCTTGCAATG GACGACCTTC GATTTCTTCG
                         6

        7             9                           11
TGAAAACATC ACCACTGGTT GTGCTGAACA CTGTTCTTTG AACGAAAACA
ACTTTTGTAG TGGTGACCAA CACGACTTGT GACAAGAAAC TTGCTTTTGT
         8                  10

     KpnI    BamHI
TTACGGTACC G
AATGCCATGG CCTAG
  12
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TABLE X

ECEPO SECTION 2 OLIGONUCLEOTIDES

Line	No.	Sequence
	1.	AATTCGGTACCAGACACCAAGGT
	2.	GTTAACCTTGGTGTCTGGTACCG
5	3.	TAACTTCTACGCTTGGAAACGTAT
	4.	TTCCATACGTTTCCAAGCGTAGAA
	5.	GGAAGTTGGTCAACAAGCAGTTGAAGT
	6.	CCAAACTTCAACTGCTTGTTGACCAAC
	7.	TTGGCAGGGTCTGGCACTGCTGAGCG
10	8.	GCCTCGCTCAGCAGTGCCAGACCCTG
	9.	AGGCTGTACTGCGTGGCCAGGCA
	10.	GCAGTGCCTGGCCACGCAGTACA
	11.	CTGCTGGTAAACTCCTCTCAGCCGT
	12.	TTCCCACGGCTGAGAGGAGTTTACCA
15	13.	GGGAACCGCTGCAGCTGCATGTTGAC
	14.	GCTTTGTCAACATGCAGCTGCAGCGG
	15.	AAAGCAGTATCTGGCCTGAGATCTG
	16.	GATCCAGATCTCAGGCCAGATACT

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