Amgen Inc. v. F. Hoffmann-LaRoche LTD et al Doc. 547 Att. 14

EXHIBIT 14

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IN THE APPEAL PROCEEDINGS AGAINST EP 148 605 OF KIRIN-AMGEN

DECLARATION OF RICHARD D. CUMMINGS, Ph.D.

I, Richard D. Cummings, do hereby declare as follows:

1. I submit this declaration in support of EP 148 605 to Kirin-Amgen, Inc. entitled "Production of Erythropoietin." I previously provided oral testimony concerning this patent at the Oral Proceedings held before the Opposition Division on November 24-25, 1992. I repeat in this declaration the same opinions provided to the Opposition Division concerning the differences between recombinant erythropoietin ("rEPO") and erythropoietin derived from natural urinary sources ("uEPO"). In addition, I have reviewed the Statements of Grounds of Appeal submitted by the four Opponents together with the supporting declarations and exhibits regarding the novelty attacks on the rEPO polypeptide claims, and I respond herein to the positions taken by the Opponents and their declarants.

2. I currently am a Professor in the Department of Biochemistry and Molecular Biology at the Oklahoma Health Sciences Center in Oklahoma City, Oklahoma, U.S.A. Previously, I was a Professor of Biochemistry at the University of Georgia and Associate Director of the Complex Carbohydrate Research Center in Athens, Georgia. Attached hereto as Exhibit A is my curriculum vitae which provides information on my educational and professional qualifications, my memberships in professional organizations, awards and honors received, and a description of the primary areas of my research. Since 1980, my research has been focused in the field of glycoproteins, with emphasis on the carbohydrate portion of the protein, including the biosynthesis, structure, analysis and biological effects of various carbohydrate structures. As shown in my

curriculum vitae, I have authored or co-authored more than 50 publications on these and related topics.

3. Summary of Opinion

3.1 Consistent with my testimony before the Opposition Division, I submit this declaration to confirm that rEPO differs from uEPO. These differences are found in the carbohydrate portion of the proteins. The fact that differences exist is not surprising because it is well known in the art that the glycosylation process in the production of a glycoprotein is host-cell specific, i.e., each different host cell has its own unique machinery for accomplishing glycosylation which results in a carbohydrate unique for that host cell. Thus, when a gene (or DNA sequence) is taken out of its natural environment and through recombinant DNA technology is introduced into a new host cell, which is then maintained under cell culture conditions, it is fully expected that the recombinantly produced protein will have a different carbohydrate than the corresponding natural protein. In some cases, the differences may be slight, but for heavily glycosylated proteins like EPO, the differences are detectable using what have become standard analytical techniques. Since the carbohydrate portion of the protein can have direct effects on the biological properties of the protein, differences in the carbohydrate between a recombinant protein as compared to the natural can result in a detectable difference in biological activity.

3.2 I have read the declarations and literature references submitted by the Opponents asserting that rEPO cannot be distinguished from uEPO. I do not agree with these arguments or the conclusions drawn by the declarants from the literature references. In fact, I believe that all of the relevant references submitted by the Opponents actually support the conclusion that the carbohydrate portion of rEPO differs from that of uEPO. Every study conducted and reported in the literature shows that there is some difference between the rEPOs and uEPO. The Opponents' declarants seem to ignore these differences in reviewing the references. Since glycosylation is

host-cell specific, the rEPOs made in different host cells will also exhibit differences between themselves, but each rEPO is still different from uEPO.

4. Background Discussion of Glycoproteins

4.1 Before reviewing the relevant literature references on erythropoietin, I believe that it might be useful to discuss in general terms what is known about glycoproteins and particularly the carbohydrate portion. A glycoprotein consists of a polypeptide backbone with one or more carbohydrate side chains covalently attached thereto. Two major classes of carbohydrate chains are known: the so-called N-linked and O-linked oligosaccharides which are linked to the polypeptide backbone via a nitrogen and oxygen respectively. N-linked oligosaccharides attach to asparagine residues found in a certain sequence in the polypeptide backbone via N-acetyl glucosamine (GlcNAc) while O-linked oligosaccharides can attach to serine or threonine residues via N-acetyl-galactosamine (GalNAc).

4.2 The carbohydrate side chains are termed oligosaccharides because they are composed of monosaccharides such as mannose, GlcNAc, galactose, fucose, GalNAc and sialic acids in covalent linkage to each other. These oligosaccharides can have different numbers of monosaccharides and can be present in different arrangements. For example, the oligosaccharide chains can be branched or unbranched; the chains can contain different numbers of monosaccharides; some chains can have repeating subclasses called extensions; or the monosaccharide groups can be linked either alpha or beta, thus resulting in different spatial arrangements. The result of this variability is microheterogeneity which simply indicates that different forms of the glycoprotein (all of which have the same amino acid backbone) are present. Again, these different forms are a result of different carbohydrate structures. Attached as Exhibit B is a figure illustrating some of the various types of oligosaccharide chains that can be present on a glycoprotein.

4.3 The process of glycosylation of a protein is a complex interaction between a host cell and the polypeptide backbone. The source of the observed microheterogeneity in oligosaccharides is the mechanism by which these structures are synthesized within the cell. In contrast to the synthesis of the amino acid backbone which is directed by a nucleic acid template, the biosynthesis of oligosaccharides is due to the chance interaction of enzymes (glycosyltransferases) which attach single sugar units to the growing oligosaccharide chain. Thus, the structure depends on the relative levels of each glycosyltransferase and the sequence of encounters with the oligosaccharide chain. As a result, one might characterize glycosylation as "a hit and run process."

4.4 Since different host cells each naturally possess different glycosylation machinery and materials, e.g., different glycosyltransferases, levels of glycosyltransferases, sugar nucleotides, etc., glycosylation is host cell specific. Thus, recombinant glycoproteins differ from their natural counterparts because the host cells used are different cells in different environments with different glycosylation abilities than the cells in which the protein is produced naturally.

4.5 Another reason that recombinant glycoproteins differ in the microstructure of the carbohydrate from the natural protein is the processing which host cells go through in order to effectively express the desired protein. The physiological status of the host cell is altered by the transfection and immortalization of the recombinant host cell, and these alterations result in a different microstructure for the carbohydrate. In addition, cell culture conditions in recombinant processes are different from the natural environment in the body which influences the glycosylation process. The macrostructure of the carbohydrate is unchanged, e.g., CHO cells continue to O- and N-glycosylate, but changes in the host cell and its environment affect the way in which the monosaccharides are added to the oligosaccharide chains. Thus, even if the natural cell could be grown in culture, e.g., human kidney cells that express EPO, the transfection,

immortalization and cell culture conditions would result in a recombinant glycoprotein that is different from the natural protein.

5. The Carbohydrate of Recombinant and Urinary Erythropoietin

5.1. Erythropoietin is a heavily glycosylated protein with three N-linked chains at positions Asn-24, -38 and -83, and one O-linked chain at position Ser-126. The carbohydrate comprises about 40% by weight of the glycoprotein and plays a role in the biological activity of the protein. For example, it was known from Dr. Eugene Goldwasser's work in 1974 that if the sialic acids are removed from uEPO, then the product exhibits very low levels of in vivo biological activity. See Goldwasser et al., J. Biol. Chem., 249, 4202 (1974) (Tab 1). This work provided the conclusion that the carbohydrate of EPO was important for biological activity. It is further known today that, to a point, higher numbers of sialic acids per molecule of EPO lead to higher levels of in vivo biological activity. (See e.g., Imai et al. Eur. J. Biochem. 194, 457,460 (1990)). The sialic acids are found at the terminal end or "cap" of the oligosaccharide chains and apparently play a role in the clearance of the protein from the body. Sialic acids can be present in various forms, including the predominant form N-acetyl-neuraminic acid (NANA or Neu5Ac) or a derivative thereof such as N-glycolylneuraminic acid (NGNA or Neu5Gc). See generally Varki et al. (Tab 2).

5.2. Several publications describe the various structures of the oligosaccharides found in rEPO and in uEPO, see e.g., Takeuchi, p. 3659 (Tab 3), Tsuda, p. 5649 (Tab 4), and the discussion of the references below. (For the convenience of the reader, an index listing is provided at the end with a full citation to the various references cited herein.). As shown in Takeuchi and Tsuda, the N-linked chains are composed of bi-, tri-, and tetraantennary structures with some chains containing one or more N-acetyllactosamine extensions or repeating units. These extensions are much more prevalent in rEPO than in uEPO. Takeuchi, p. 3659 and Tsuda, p. 5649.

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The Patent and the Literature References Demonstrate a Difference between rEPO and uEPO

6. In view of the Opponent's arguments and the reliance placed by them on the cited literature references, I would like to review how EP 148 605 and the literature references actually show rEPO to be different from uEPO.

6.1 EP 148 605

First, the disclosure of EP 148 605 reports on differences between rEPO and uEPO as evidenced by using SDS-PAGE and Western blot analysis. SDS-PAGE is a separation technique in which proteins are forced to migrate by an electric field through a gel of defined porosity. The negatively charged detergent SDS binds to the polypeptide backbone and imparts a negative charge to the protein. The mobility of the protein through the gel is a reflection of the size of the protein and other negative charges found on the protein such as those contributed by sialic acids and sulfates on the carbohydrate. Although the mobility of the protein through the gel is used for estimating the molecular weight of a protein by comparison to the mobility of proteins of known molecular weight, the influence of other negative charges may modify this value for a particular protein. Therefore, the molecular weight determined by SDS-PAGE is indicated as "apparent". Western blot is a detection technique which is applied to proteins separated by SDS-PAGE by using a specific antibody to identify the protein of interest.

6.2 On page 29, lines 6-16, EP 148 605 (Tab 5) indicates that a preliminary attempt was made to characterize rEPO expressed in CHO cells and in COS-1 cells by comparing them to a pooled human urinary extract using SDS-PAGE and Western blot. By these techniques, both the CHO and COS-1 expressed rEPO showed a higher apparent molecular weight as compared to uEPO. After treating all three samples with an enzyme (Neuraminidase) to remove the sialic acids, both asialo rEPOs were still larger than the asialo uEPO. Treatment of the samples with the Endoglycosidase F

enzyme (to remove the N-linked carbohydrate) resulted in the CHO rEPO and uEPO having "essentially identical molecular weight characteristics." This shows that the differences seen in the undigested samples of rEPO and uEPO were due to differences in the carbohydrate. As discussed below, similar differences between rEPO and uEPO were shown using SDS-PAGE analysis by several other groups: Yanagi, p. 422 (Tab 6); Sasaki, (1987) p. 12061 (Tab 7); and Imai, p. 354 (Tab 10).

7. Storring and Gaines Das (1992) (Tab 8)

7.1 From a review of the Opposition Division's decision, I notice that the Storring and Gaines Das article is cited by the Division, and properly so, to support the finding of a difference between rEPO and uEPO. This publication resulted from an international study to establish the "International Standard for Recombinant DNA-derived Erythropoietin" as reflected in the title. This collaborative study compared four rEPOs provided by Amgen, Boehringer-Mannheim, Behringwerke, and Snow Brand with urinary EPO provided by Snow Brand (EPO A) and Chugai (EPO B). The rEPO samples were prepared using three different host cells: Chinese hamster ovary (CHO), baby hamster kidney (BHK), and the C127 mouse fibroblast cell line. The uEPO samples were purified according to methods described in Yanagawa (1984) (EPO A) and Imai (1990) (EPO B). In addition, two serum EPO samples were studied for charge and immunoreactivity.

7.2 The conclusion of this study was stated by the authors as follows:

> Significant differences in in-vivo and in-vitro biological, immunological and physicochemical properties were found between these four rDNA EPO preparations and between them and the HU EPO in the two purified preparations and in the 2nd IRP. There were also differences between the immunoreactivities of the two serum EPO samples included in the study, and between them and the immunoreactivities of the purified EPOs. The differences between rDNA EPOs appeared to be related to differences between the cells used for their biosynthesis, but may also be the result of differences in purification methods and inter-batch variations. (Storring, Tab 8, p. 459).

7.3 The authors anticipated that the carbohydrate structures and the isoform compositions might differ between the various EPO samples due to the general proposition I outlined above, i.e., that glycosylation is host cell specific, so that the rEPOs would differ between themselves and that each would be different from uEPO:

> "It was anticipated that the carbohydrate structures and therefore the isoform compositions might differ between rDNA EPO and extracted naturally occurring EPO, between rDNA EPO synthesized in different cell lines, between EPO produced by different laboratories and perhaps also between batches of EPO from the same laboratory (Storring, 1987). The glycosylation of the translated polypeptide depends on the nature and activities of the glycosylation systems which differ with the cell-type (Kornfeld & Kornfeld, 1985) and with its physiological status (Taylor & Weintraub, 1985)." (Storring, Tab 8, p. 460).

7.4 From my review of the literature, I certainly agree with the authors that "[t]his collaborative study provides the most comprehensive comparison available for EPO, or any other glycoprotein hormone, between rDNA products from different laboratories and synthesized in different cell lines, and between them and the extracted naturally occurring

hormone." (Storring, Tab 8, p. 460). In fact, it is further noted that 26 laboratories from 11 countries took part in the study.

7.5 Storring and Gaines Das found differences between rEPO and uEPO in terms of (1) electrophoresis and isoelectric focusing, (2) in-vivo bioassays and (3) immunoassays. Due to these differences, the authors suggested that a separate international standard be adopted for rEPOs than for uEPOs. These differences are discussed briefly below.

7.6 Isoelectric Focusing: The results of the isoelectric focusing are shown in the gel exhibited on page 473, Figure 4. EPO-A which is a uEPO preparation is in lane 1, rEPO preparations are in lanes 2-5, and EPO-B which is allegedly a purified form of uEPO is shown in lane 6. From my review of the gel, the four rEPOs are distinctly different from the two uEPOs. EPO-A is clearly more acidic or highly charged than the rEPOs as evidenced by the fact that it travels further down the gel. EPO-B shows the presence of the same types of isoforms as the rEPOs (which are delineated by each bead-like grouping on the gel) but from the intensities of the spots on the gel, it is readily seen that the relative amounts of each isoform, or distribution of the isoforms, in EPO-B are different from those present in each of the rEPOs. The authors state this very fact:

> "In all preparations except EPO-A, the major and minor components, where present in more than one preparation, had similar isoelectric points. But, there were also differences between all these preparations in the proportions and/or types of EPO isoforms present. EPO-A differed most markedly from other preparations in containing isoforms which tended to be less discrete and more acidic." (Storring, Tab 8, p. 473)

Based on this clear statement and on the gel itself, I do not understand how the Opponents and their declarants can argue that this Figure 4 shows rEPO to be identical to uEPO. In

fact, Figure 4 shows them to be different even in comparing EPO-B to the rEPOs.

7.7 In discussing the comparison of the rEPOs and the uEPOs by electrophoresis and isoelectric focusing, the authors agree with my conclusion:

> "IEF and/or zone electrophoresis showed differences between the EPO isoform compositions of all six purified EPOs and between them and the 2nd IRP. These differences were smaller between the four rDNA EPOs than between them and the HU EPO in EPO-A and the 2nd IRP, which contained a greater proportion of more acidic isoforms. (The differences between EPO-A and EPO-B presumably reflect differences in the methods used to isolate these preparations.) The more acidic isoforms found in EPO-A and the 2nd IRP appear to be more representative of those in unextracted serum and urine. (Wide & Bengtsson, 1990; Tam, Coleman, Tiplady et al. 1991) than are the EPO isoforms in EPO-B and in the rDNA EPOs. The findings of this study together with those of Wide & Bengtsson (1990) and Tam et al. (1991) indicate that both extracted and unextracted human urinary EPO contain more acidic isoforms than unextracted serum EPO." (Storring, Tab 8, p. 474-75.)

7.8 The authors concluded that EPO-A was more representative of uEPO because of the presence of more acidic forms which might be expected in a protein extracted from urine. EPO-A was purified from urine using immunoprecipitation as described by Yanagawa (1984) (Tab 9) while EPO-B was purified as described by Imai (1990) (Tab 10). The Imai process include steps differing from the Goldwasser purification process as published in Miyake et al. (1977) as well as the use of reverse phase HPLC to supposedly further purify the sample. From my review of the literature, I concur that EPO-A, based on electrophoretic mobility, appears to be a better representation of the types of isoforms in uEPO than that prepared

by Imai. Certainly, EPO-A is closer to the type of uEPO produced by the Miyake purification process as is confirmed by the analytical results discussed in the Strickland declaration.

7.9 The results of the bioassays and the immunoassays also showed a difference between rEPOs and uEPO. The findings from the bioassays were "consistent with other reports in the literature of significant differences in the in-vivo and/or in-vitro specific bioactivities between HU and rDNA EPO (Recny et al., 1987; Goto et al., 1988; Imai et al., 1990) and between rDNA EPOs synthesized in different cell lines (Goto et al., 1988)." (Storring, p. 476) Similarly, the "HU EPOs differed from the rDNA EPOs in their immunoreactivities." (Storring, p. 476).

7.10 Storring and Gaines Das indicated that the basis for the differences between the activities of the rEPO and uEPO preparations was the differences in glycosylation:

> The differences in the bioactivities between these seven preparations of rDNA and HU EPO are in turn probably a reflection of their differing carbohydrate structures and isoform compositions. Thus differences in, or modifications of, the carbohydrate structure of EPO have been shown to influence markedly its bioactivity whether measured in-vivo or in-vitro [citations omitted]." (Storring, p. 476).

7.11 This comprehensive study shows the results of a number of analyses all of which indicate a significant difference in the carbohydrate of rEPO as compared with uEPO. These conclusions are wholly consistent with the other literature references which are in fact cited in the Storring article. There is simply no evidence in Storring to support the conclusion that rEPO is identical to uEPO as argued by the Opponents.

8. Tsuda et al (1988) (Tab 4)

8.1 In this article, the authors report a comparative study of N-linked oligosaccharides found on uEPO and rEPO. The rEPO was prepared using a genomic clone in BHK cells while the uEPO was prepared as described by Yanagawa et al. (1984) (Tab 9). The authors reported that the results from BHK produced EPO "were similar in many respects" to CHO cell produced rEPO, although there were some differences. More importantly, the authors "noticed, however, significant differences between uEPO and rEPO (BHK); e.g., the tetraantennary structure with an N-acetyllactosaminyl repeat that existed in rEPO (BHK) was barely detectable in uEPO." (Tsuda, p. 5647). They also found that rEPO possesses a higher content of sialic acid than uEPO. (p. 5648)

8.2 Looking at the data presented in Tsuda, Figures 1 and 2 show a comparison of HPLC profiles between two samples of uEPO (panels a and b) and rEPO from BHK cells (panel c). One can readily detect the difference in HPLC profiles which is indicative of a different structure for the BHK rEPO as compared with either uEPO preparation. Figure 3 (p. 5649) shows the various structures of asialo N-linked oligosaccharides and the relative amounts of each for the two u-EPO preparations and the BHK rEPO. Again, it is apparent that the isoform distribution is different between rEPO and the two uEPO preparations. As illustrated, the N-acetyllactosamine structure, Gal(ß1-4) GlcNAc (ß1-3), repeats and thereby extends the oligosaccharide chains in rEPO while their presence was undetected in both uEPOs. This difference was also noted by several other groups as discussed below.

9. Yanagi, et al. (1989) (Tab 6)

9.1 This article describes an attempt to produce rEPO that contained carbohydrate chains closely resembling naturally occurring EPO by using a human cell line called Namalwa cells, which is a human B-lymphoblastoid cell line, to express rEPO. Even using a human host

cell, however, the results of the study showed that rEPO was discernibly different from uEPO. The uEPO analyzed was prepared as described in Yanagi et al. (1987). As seen from Figure 2B, page 22, of the 1989 article, rEPO from Namalwa cells exhibited a more diffused band with a higher molecular weight than did uEPO on SDS-PAGE, thus clearly indicating that the two samples were not identical. The authors excluded the possibility that the rEPO was impure because of the use of reverse-phase HPLC in which the rEPO eluded as a single peak. Rather, they concluded that the heterogeneity observed in Namalwa EPO was likely due to the abundance of "bulky oligosaccharide chains." (p. 425).

9.2 HPLC profiles (Figure 5) of the pyridylamino derivatives of the oligosaccharides with the sialic acids removed show a clear difference between rEPO and uEPO. The authors reported more than 20 peaks of intact pyridylamino oligosaccharides. While both rEPO and uEPO include most of these structures, the relative amounts of each type of structure are different as evidenced by the different relative heights of corresponding peaks on the HPLC profiles. The authors reported that some peaks found in the rEPO profile were not present at all in the uEPO profile indicating that rEPO as produced in Namalwa cells contained some oligosaccharide structures not found in uEPO:

> "Although several extra peaks were found in the latter part of the chromatogram of Namalwa EPO, suggesting that N-linked oligosaccharides of Namalwa EPO were more heterogeneous than those of urinary EPO, the rest of the peaks emerged in common between Namalwa and urinary EPOs. ... It is thus most likely that the content of each oligosaccharide differs, but almost all oligosaccharides of Namalwa EPO are also found in naturally occurring EPO in common, although the possibility still remains that urinary excreted EPO is not valid as representative of the natural hormone." (p. 425).

10. Sasaki et al. (1987) (Tab 7)

10.1 This paper provided one of the first analyses of the carbohydrate structure of rEPO and a comparison with uEPO. Dr. Fukuda who submitted a declaration in this proceeding on behalf of Opponent IV is a co-author on this paper. In the abstract, the authors report that "the carbohydrate moiety of urinary EPO is indistinguishable from recombinant erythropoietin except for a slight difference in sialylation, providing the evidence that recombinant erythropoietin is valuable for biological as well as clinical use." (p. 12059, emphasis added). In light of the data presented in the article which does show a distinct difference in the carbohydrate between rEPO and uEPO, as a scientist, I can only interpret the use of such phrases as "indistinguishable" as used here, and elsewhere by others, in the expected clinical context rather than in a structural or compositional meaning. That is to say, I believe that it was surprising that rEPO would be as similar as it is to uEPO, and the similarity enhanced the expectation at that time that rEPO would prove to be useful clinically.

10.2 The urinary and recombinant EPO studied in this article were both purified using a procedure "slightly modified from that of Miyake et al., and fractionation on a Vydac C4 reverse-phase HPLC column ... was included." (p. 12059) Even using this same supposed enhanced purification for both samples, rEPO still was shown to be different from uEPO.

10.3 A review of the data reported in the article confirms a difference between the carbohydrate portion of rEPO and uEPO. For example, Figure 1, page 1201, shows an SDS-PAGE gel for rEPO (lane A) and uEPO (lane B) with the rEPO band being wider and exhibiting a higher molecular weight (i.e., slower mobility) than the uEPO band. Figure 7, page 12070, shows the ion-exchange HPLC of N-linked saccharides from rEPO (panel A) compared with those of uEPO (panel B). Again, I believe that the existence of differences between

rEPO and uEPO as shown by the differences in the HPLC profiles is easily discernible. The relative distribution of the peaks on the two profiles is different, and some peaks in rEPO do not appear to be present at all in the uEPO profile. The oligosaccharides from uEPO (Figure 7, panel B) clearly include more acidic species as they eluted in later fractions from the ion-exchange HPLC than the oligosaccharides from rEPO (panel A).

10.4 In his declaration, Dr. Fukuda refers to the mass spectrometry analysis reported in Sasaki as showing no evidence of sulfated oligosaccharides or of α2-6 linked sialic acids in uEPO. I believe this to be an error. First, mass spectrometric data does not provide sufficient information without the use of appropriate standards to identify all component monosaccharides and their linkages. Other chemical evidence is necessary to provide a complete chemical structure for an oligosaccharide. Second, Sasaki reports this structural analysis only for rEPO, see Table III, pp. 12064-67, but there is no indication that a similar analysis was conducted on uEPO. Without more, it is simply a guess to determine the presence or absence of sulfates or α2-6 linked sialic acid in uEPO by mass spectrometry.

10.5 In simply comparing the results of the mass spectrometry for rEPO and uEPO as reported in Sasaki, one can discern differences. For example, fragment ions at 418 and 432 were observed for uEPO (Figure 3B) but not for rEPO (Figure 3A). In addition, the mass spectrometry data in Figure 10 shows both quantitative and qualitative differences in fragment ions observed for O-linked oligosaccharides.

10.6 From these results, I would not consider rEPO and uEPO to be "indistinguishable", but rather, the study shows distinct structural differences between the N-linked and O-linked chains of rEPO as compared with uEPO as well as a difference in the number of sialic acids attached to the oligosaccharide chains. Table III shows the relative amounts of asialo N-linked saccharides for rEPO, no

structural analyses being reported for uEPO, and the data shows a "large proportion" of tetraantennary saccharides with one (32.1%), two (16.5%) and three (4.7.%) N-acetyllactosamine repeats. As indicated above, Tsuda and others have reported very little, if any, repeating units found in uEPO. The authors also reported another difference in that uEPO lacks bi-antennary saccharides (p. 12071). Thus, differences are shown between rEPO and uEPO even when they are purified by the same process.

11. Takeuchi et al. (1988) (Tab 3)

11.1 In this study, rEPO produced from CHO cells was compared with uEPO purified using the first two steps of the Miyake procedure coupled with immunoprecipitation using a monoclonal antibody. The authors found differences between rEPO and uEPO in oligosaccharide distribution as illustrated in Table II, page 3659. The authors noted differences in the amount of oligosaccharides with N-acetyllactosamine repeating units (34.5% for rEPO and only 7.5% for uEPO). Also, the authors concluded that all the sialic acid residues in rEPO produced in CHO cells are attached via the α2-3 linkage to Gal, while only about 60% of the sialic acid linkages in uEPO are of the α2-3 Gal type with the remainder being α2-6 Gal linked. These differences in linkage were attributed to different levels of glycosyltransferases present in the host cells. (See p. 3660) The authors also suggest the presence of sulfated oligosaccharides. (See p. 3658)

12. Imai et al. (1990) (Tab 10)

12.1 Although the authors again conclude that the results "demonstrate that rhEPO is indistinguishable from uEPO, and so is valuable for further extensive research and for clinical purposes," (p. 356) the data presented in the article does illustrate differences. The uEPO was prepared using a process differing from that disclosed in Miyake and included the use of a reverse-phase column (twice). This is the process reportedly used to prepare the EPO-B sample discussed in

Storring. There are no details given for the process used to purify the rEPO except to say that it was "purified to homogeneity in a similar manner." (p. 353) The changes to the Miyake process were claimed to have some effect because the authors report a higher specific activity for uEPO and cite to the absence of denaturing agents as the reason for the increase in activity:

> "In the purification methods employed here denaturing agents, which have been used for the purification of uEPO, were not used to prevent loss of the activity in vivo." (p. 354).

Interestingly, the increase in specific activity was not attributed to the use of reverse phase HPLC in addition to the Miyake procedure.

12.2 Looking at the data, Figure 1 shows a difference on SDS-PAGE with rEPO (lanes 1 & 2) being slightly higher than the corresponding uEPO preparations (lanes 3 & 4). Figure 2 shows an isoelectric focusing gel and illustrates a much broader band for uEPO (lane 2, Figure 2A) than for rEPO (lane 3). In comparing the biological activities, the authors report that rEPO has a significantly higher specific activity as compared with uEPO. In the supplemental materials, Figures 2 and 3 show a difference in HPLC profiles between the peaks for rEPO and uEPO using reverse phase HPLC.

13. Nimtz et al. (1993) (Tab 11)

13.1 This article reports on the carbohydrate structure of rEPO expressed in BHK-21 cells as produced by Elanex and Merckle. In the text, the authors report that the oligosaccharide structures of BHK produced EPO "were found to be similar to those from urinary EPO." However, the article and the data presented disclose several differences. First, it is reported that only about 60% of the protein from BHK cells was found to be O-glycosylated whereas it is known that urinary EPO (as well as rEPO from CHO cells) is nearly 100% O-glycosylated. See Browne et al., Cold Spring Harbor, 51, 693,698

(1986) (Tab 12). The BHK produced EPO is also reported to contain more sialic acids than CHO produced EPO which in turn has more sialic acids than urinary EPO. Specifically, half of the tetrantennary oligosaccharides from BHK cells were found to be fully sialylated, 36% contain 3 sialic acid residues and only about 13% are disialylated. Concerning the linkage, the authors report that the oligosaccharides were more than 97% α 1-6 fucosylated at the proximal GlcNAc and that the sialic acid was exclusively linked α2-3 to Gal. It is known from Takeuchi that uEPO contains sialic acid in both α2-3 and α2-6 linkages to Gal and that about 82% of the oligosaccharides are fucosylated. Finally, Nimtz reported terminal GalNAc-GlcNAc structures which have not been reported for uEPO.

14. Wide and Bengtsson (1990) (Tab 13)

14.1 In this article, the authors report on a study of the charge heterogeneity of human serum erythropoietin by measuring mobility of serum by zone electrophoresis followed by radioimmunoassay of EPO. The study compared serum EPO with rEPO supplied by both Boehringer Mannheim and Kirin-Amgen and the 2nd IRP for EPO which I understand is an International Reference Preparation of uEPO.

14.2 The authors concluded:

> The recombinant EPO preparations had a median charge which was much less negative than that of the 2nd IRP for EPO and of the EPO in serum in healthy individuals. As the polypeptide chain of recombinant EPO is claimed to be identical with that of human EPO, it seems likely that the differences are due to different degrees in glycosylation. EPO in blood specimens taken 24 h. after s.c. [subcutaneous] injection of recombinant EPO had a median charge which was close to that of the recombinant EPO preparation and far from that of EPO in a serum specimen taken before the injection. The results suggest that the methods used in this investigation may be useful for detecting the presence of recombinant

> EPO in the blood of persons with a normal endogenous EPO production. (Wide, p. 126)

The authors apparently believed there was a sufficient charge difference to allow the detection of rEPO over normal serum EPO. As indicated, the authors also reported that the uEPO preparation was more acidic (i.e., more negative) than the rEPOs.

15. Strickland Declaration

15.1 I have reviewed the declaration of Dr. Thomas W. Strickland, which I understand is being submitted herewith, concerning both Dr. Strickland's work in comparing rEPO with uEPO using isoelectric focusing and his sulfation studies. Upon reviewing Dr. Strickland's results, I concur with his conclusions that 1) uEPO as purified by the Miyake process exhibits a lower or more acidic isoelectric point as compared to rEPO and 2) the oligosaccharides of uEPO are much more highly sulfated (over 50%) while only a small fraction of rEPO oligosaccharides (about 3%) have a sulfate group as part of the chain. One can readily see the differences between rEPO and uEPO by comparing the HPLC profiles of the respective oligosaccharides in Figures IV-VI of Exhibit TWS-3.

16. Table of Differences between rEPO and uEPO

16.1 While additional references might be consulted for further support of the above-discussed differences between rEPO and uEPO, as well as providing evidence of additional differences, I believe the references cited so far establish a number of detectable differences between rEPO and uEPO. These differences are summarized in the Table below with the supporting references being cited:

SUMMARY OF DIFFERENCES BETWEEN rEPO and uEPO	
Differences	**References**
1. SDS-PAGE analysis shows a difference	EP148 605 p.29, lines 6-16, Yanagi, p. 422, Sasaki, p. 12061, Imai, p. 354
2. uEPO is distinguishable from rEPO on isoelectric focusing (IEF) and zone electrophoresis	Storring, pp. 473-74 Strickland Decl. Wide, p. 126
3. HPLC analyses show different profiles indicating different oligosaccharide structures	Yanagi, p. 424 Sasaki, p. 12063, 70 Strickland Declaration
4. rEPOs show different linkages within the oligosaccharide chains and to the terminal sialic acids as compared with uEPO	Takeuchi, p. 3657 Nimtz, p. 39
5. rEPOs have a different percentage of extensions or repeating structures within the oligosaccharide chains	Tsuda, p. 5659 Takeuchi, p. 3659 Sasaki, p. 12064-67
6. rEPOs have a different oligosaccharide distribution than uEPO for N-linked chains	Tsuda, p. 5649 Takeuchi, p. 3659
7. rEPOs have a higher number · sialic acids	Sasaki, p. 12059, 70 Nimtz, p. 54
8. rEPOs have more highly bran...ed oligosaccharides than uEPO.	Tsuda, P. 5649 Takeuchi, p. 3659
9. rEPOs have higher biological activity	Imai, p. 356 Storring, p. 476
10. uEPO has a much higher percentage of sulfated oligosaccharides than rEPO	Strickland Declaration

17. Response to the Declarations of Drs. Conradt, Nimtz and Fukuda

17.1 I have reviewed the declarations of Dr. Harald S. Conradt submitted by Behringwerke, Dr. Manfred Nimtz submitted by Merckle, and Dr. Minoru Fukuda submitted by Boehringer Mannheim. As I indicated, I disagree with their conclusions and with their characterizations of the literature references. I believe the differences between rEPO and uEPO are clear from the references discussed above, many of which are cited by the Opponents' experts. With all due respect, the Opponents' experts tend to focus on one isolated teaching from each of the references to argue that a cited difference may not be used to distinguish every rEPO from every possible uEPO while ignoring the overwhelming evidence in the references that differences do exist. Also, in many cases, their conclusions are improper based on the references they cite.

17.2 Dr. Conradt begins his analysis by citing Tsuda and Yanagi for support that uEPO preparations are different, and therefore, no true "yard stick" uEPO preparation exists. I do not understand the relevancy of Tsuda and Yanagi for this point since I understood that the Opponents were arguing the uEPO purified according to Miyake was the appropriate prior art. Neither Tsuda nor Yanagi disclose any analysis of Miyake uEPO. The Tsuda article does compare rEPO with uEPO prepared according to Yanagawa (1984). The immunoprecipitation process of Yanagawa is the same process as reported in Sasaki (1983), (Tab 14) which I am informed is being asserted by one Opponent as prior art to EP 148 605. Regardless of that, the real relevancy of Tsuda and Yanagi is what Dr. Conradt ignores, i.e., the differences disclosed in Tsuda and Yanagi for each of the uEPO preparations as compared to the different rEPOs. (See discussion of these references above). These side-by-side comparisons of uEPO and rEPO show the carbohydrates to be different regardless of what uEPO or rEPO preparation is selected for analysis.

17.3 The Opponents' experts argue that the five differences between uEPO and rEPO discussed before the Opposition Division do not apply to every rEPO or uEPO studied in the literature. As I understood it, these five cited differences were simply examples of the differences reported in the literature. I have included additional differences drawn from the literature in my Table above. These differences may not all apply to every rEPO, but every rEPO will show some differences as compared with uEPO. I believe that my discussion of the references above has illustrated that point, i.e., that every rEPO shows some difference to uEPO. The opponents do not argue to the contrary but seem to ignore the differences which are plainly presented in the references. Moreover, I do not believe that the conclusions reached by the Opponents' experts are at all supportable especially in light of the full teachings of the cited references. I will briefly reply to the arguments raised by the Opponents regarding the five differences discussed before the Opposition Division.

17.4 SDS-PAGE Analysis

17.4.1 Both Drs. Conradt and Nimtz cite to Storring for support that the rEPO and uEPO preparations migrate "in the same molecular weight range." They conclude therefrom that rEPO is identical to uEPO. I consider that simply the use of the term "range" indicates that the products showed some differences while being in a similar proximity. It certainly does not imply that rEPO and uEPO are identical as argued, and without looking at the gels, one cannot understand what is meant by the phrase "same range." As displayed in several other references, e.g., EP 148 605, Yanagi, Sasaski (1987), and Imai, analysis by SDS-PAGE shows a difference between rEPO and uEPO even though they might be considered to be in the same range. While it is true, as suggested by Drs. Nimtz and Conradt, that many factors influence the movement of glycoproteins on SDS-PAGE, there is absolutely no evidence that any of these factors affected all these side-by-side comparisons of rEPO and uEPO conducted by at least four different independent groups. Both

Drs. Nimtz and Conradt speculate that the band widening for rEPO as shown in Sasaki is caused by different amounts of protein being applied. There is no basis for this speculation especially in view of the conclusion by the authors that there is a difference in sialylation between uEPO and rEPO. Such a difference would be reflected in a different band width on SDS-PAGE. Drs. Conradt and Nimtz both acknowledge that the migration of proteins in SDS-PAGE is influenced by the presence of sialic acids. Dr. Fukuda, a co-author on the Sasaki paper, did not argue this point and did not provide any basis for the suppositions drawn by Drs. Nimtz and Conradt.

17.4.2 Dr. Conradt cites to two articles by Egrie et al. which show several SDS-PAGE and Western blot analysis on rEPO and uEPO. Again, the gels in these articles show that the rEPO and uEPO samples migrate to similar regions, but they do not precisely comigrate. The gels would suggest the samples were similar but not identical, and any comments in the articles must be interpreted with the gels in view.

17.5 Sialic Acid Linkages

17.5.1 As I indicated above, each type of recombinant host cell has its own unique features including a unique set and amounts of glycosyltransferases which are the enzymes that accomplish the carbohydrate linkages. Thus, CHO cell produced rEPO has its own particular linkages, i.e., only α2-3 linked sialic acid. BHK and C127 cell produced rEPO will have slightly different linkages. None of these rEPOs have sialic acid linkages which have been shown to be identical to uEPO in all respects. Takeuchi found 60% of sialic acid linkages in uEPO were of the 2-3 type with the remaining 40% of the sialic acids being 2-6 linked. Dr. Conradt submitted a Table (his Exhibit 13) purporting to show methylation analysis for rEPO from C127 cells. Without a greater explanation of the Table, the results are not clear, but the ratio of 2-3 to 2-6 is not "about 2.5:1" as stated, but from Dr. Conradt's notations on the Table, the ratio appears to be 80-85% 2-3 linkages and 15-20% 2-6 linkage.

17.5.2 More importantly, however, is the fact that Dr. Conradt's Table does not provide any information regarding sialic acid linkages. The structures noted by Dr. Conradt as evidencing the 2-3 and 2-6 linkages (2,4,6-Tri-O-methyl and 2,3,4-Tri-O-methyl structures respectively) are not necessarily due to sialic acid linkages. The 2,4,6 Tri-O-methyl structure could also be due to the presence of polylactosamine extensions without sialic acids. Similarly, the 2,3, 4-Tri-O-methyl structure could be due to branching in the oligosaccharide chains and not sialic acids. Also puzzling is the presence of detectable amounts of 4,6 Di-O-methyl and 2,4 Di-O-methyl neither of which would one expect to be generated from structures known for any rEPO. Thus, the analyses must be questioned and certainly does not show that the sialic acid linkages are the same for the C127 cell produced rEPO as compared with uEPO.

17.5.3 I address Dr. Fukuda's arguments regarding the mass spectrometry in Sasaki in paragraph 10.4 above.

17.6 Isoelectric Focusing

17.6.1 Drs. Nimtz and Conradt both acknowledge that the EPO-A shown in Storring is much more acidic and exhibited a significantly different IEF pattern as compared to the four rEPO preparations. Dr. Nimtz also agrees with my interpretation that the four rEPOs show a different pattern from EPO-B, and both Drs. Nimtz and Conradt agree that Imai shows rEPO to be different from uEPO by isoelectric focusing. Quite incredibly, Dr. Conradt, and several of the Opponents in their briefs, argue that "EPO-B rather than EPO-A reflects a representative u-EPO preparation." Storring and Gaines Das concluded just the opposite. In light of the Wide study and the Strickland work, I am also of the opinion that EPO-A is more representative of uEPO than EPO-B. In any event, both Storring and Imai show EPO-B to be different by isoelectric focusing from rEPO.

17.7 Sulfation

17.7.1 Takeuchi and Strickland report side-by-side comparative studies that demonstrate uEPO has more sulfate groups attached to the oligosaccharides than rEPO. The Strickland studies, as acknowledged by Dr. Fukuda, reliably demonstrate that the difference seen between rEPO and uEPO is due to sulfation by properly identifying a peak on HPLC labeled by ^{35}S-sulfate. There is no possibility that the differences demonstrated by Strickland are due simply to the presence of N-acetyllactosamine groups as suggested by Dr. Fukuda. (Even if the dramatically different HPLC profiles were due solely to N-acetyllactosamine groups, this would be a clear difference between uEPO and rEPO). In his own studies, as described in Sasaki, Dr. Fukuda reports that they found no evidence of sulfation, but this is misleading because there is no indication that they even considered or looked for evidence of sulfation. Mass spectrometry does not provide quantitative evidence of the presence or absence of sulfation. In fact, mass spectrometry has not been used to date for the structural elucidation of sulfated N-linked oligosaccharides due to the unavailability of any standards.

17.7.2 Dr. Conradt submits another table from an unidentified Nimtz et al. publication purporting to show HPLC analysis of rEPO from C127 cells (his Exhibit 15). Dr. Conradt concludes that peaks 9 and 10 are monosulfated and "presumably" disulfated N-glycans with no explanation given that would support this conclusion. It appears to me that a number of different components are present in peaks 9 and 10 and there is no reason to conclude that any or all of them are sulfated or to what extent. Without the availability of standards to compare peaks, one cannot properly interpret Dionex data.
Dr. Conradt also states that the oligosaccharides were exhaustively digested with neuraminidase which removes the sialic acids. Yet, the Table in his Exhibit 15 labels peaks 4, 6 and 7 as disialo, trisialo and tetrasialo-N-glycans respectively thus indicating that the sialic acids are still attached to more than half of the oligosaccharides. Consequently, the methodology of this experiment and certainly the

conclusions drawn therefrom that about 20-30% of the oligosaccharides are sulfated must be seriously questioned. Further, no comparative study of uEPO using a similar methodology was conducted.

17.7.3 Dr. Nimtz cites to Tsuda and their results that "less than about 10% of the total carbohydrates of the original u- and r-EPO (BHK) remain undigested." Dr. Nimtz then jumps to the conclusion that these must be sulfated oligosaccharides and that uEPO and rEPO are sulfated to the same extent. There is, however, no rational or scientific connection between undigested oligosaccharides after the use of pepsin and glycopeptidase as reported by Tsuda and the extent of sulfation of the oligosaccharides. Sulfation has not been shown to render oligosaccharides incapable of digestion by these enzymes. Indeed, Antonsson, et al. J. Biol. Chem., 266, 6859-61 (1991) (Tab 15) used N-glycosidase F to remove sulfated N-linked oligosaccharides from the glycoprotein fibromodulin, which has oligosaccharides that are keratan sulfate.

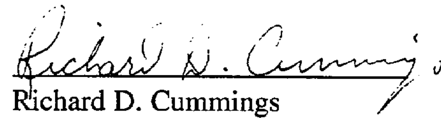
17.8 Sialic acid derivatives

17.8.1 The Hokke et al. (Tab 16) study found both sialic acid derivatives N-glycolylneuraminic acid (Neu5Gc) and N-acetylneuraminic acid (Neu5Ac) in CHO cell produced rEPO. Varki et al. (1992) (Tab 2) states in his review of sialic acids (Table I) that Neu5Gc is found "in most animals except humans." So far, Neu5Gc has been reported to be present in some gangliosides from human tumor cells, but not from normal human cells nor in glycoproteins even from the tumor tissue. See Higashi, et al. (1985) (Tab 17). Neu5Gc has never been found in uEPO or any other natural human glycoprotein. The Opponents' declarants suggest that if enough uEPO was available to analyze then this derivative might be found. Based on the lack of any evidence of such derivatives in normal human cells, that suggestion is only speculation.

18. Conclusion

In summary, I believe that rEPO is different from uEPO and that difference is found in the carbohydrate. The process of glycosylation is host cell specific, and since rEPO is expressed in a cell that is different from the cell that expresses the natural protein, the carbohydrate on any rEPO will be different from uEPO. Every study that compares a rEPO with a uEPO shows some difference. Certainly, the uEPO prepared according to the process of Miyake and Sasaki (1983) have been shown to be distinctly different from all rEPOs.

Executed this Sixth day of January 1994 at Oklahoma City, Oklahoma.



Richard D. Cummings