Exhibit 34B

to the Declaration of Cullen N. Pendleton in Support of Amgen's Opposition to Roche's Motion for Summary Judgment that Claim 7 of the '349 Patent is Invalid Under 35 USC §112 and is Not Infringed

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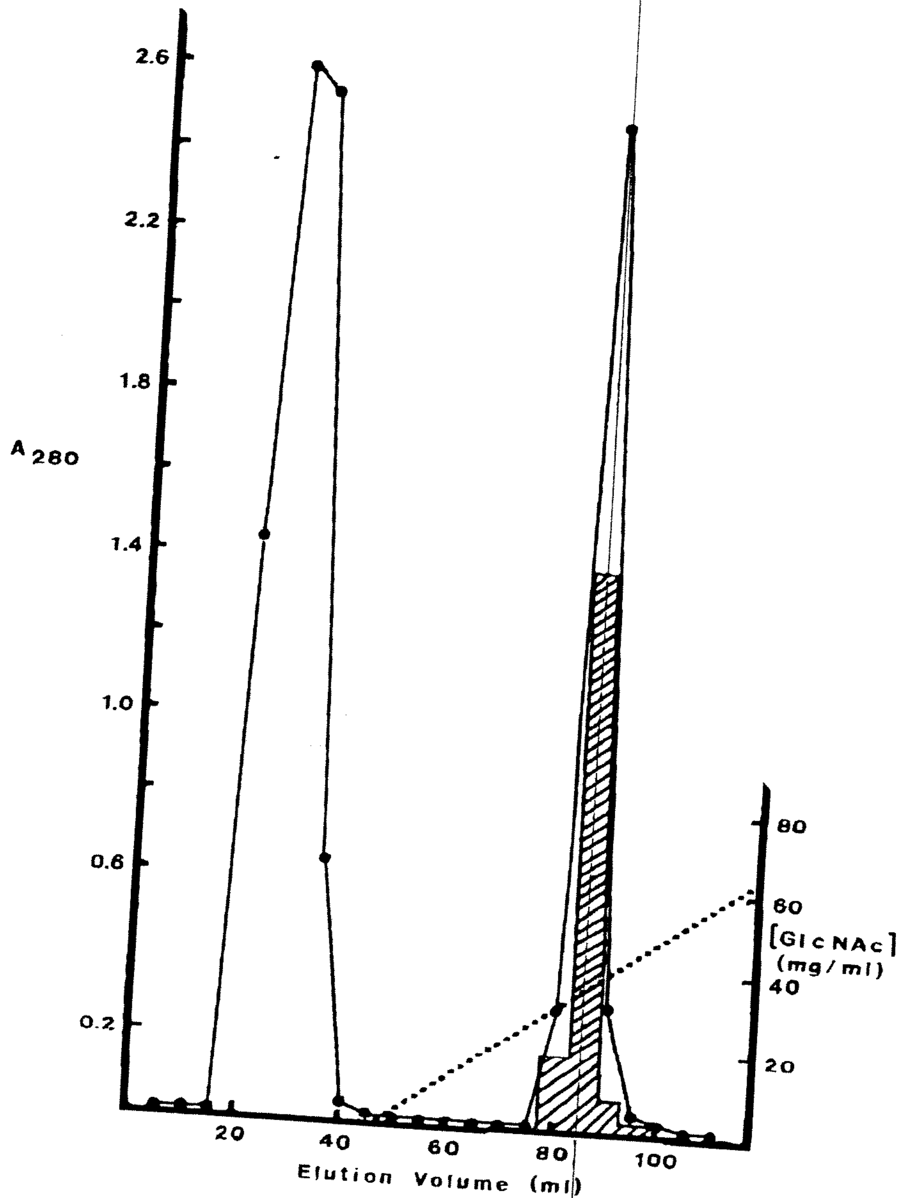

Figure 6. Affinity chromatography of wheat germ extract on a Selectin 1 column containing immobilized N-acetyl-D-glucosamine conjugated to agarose gel. The elution pattern was developed with 0.05 M acetate buffer, pH 4.5, and then with a linear gradient of N-acetyl-D-glucosamine in the same buffer. Hatched area indicates the amount of O+ RBC agglutinating activity found.

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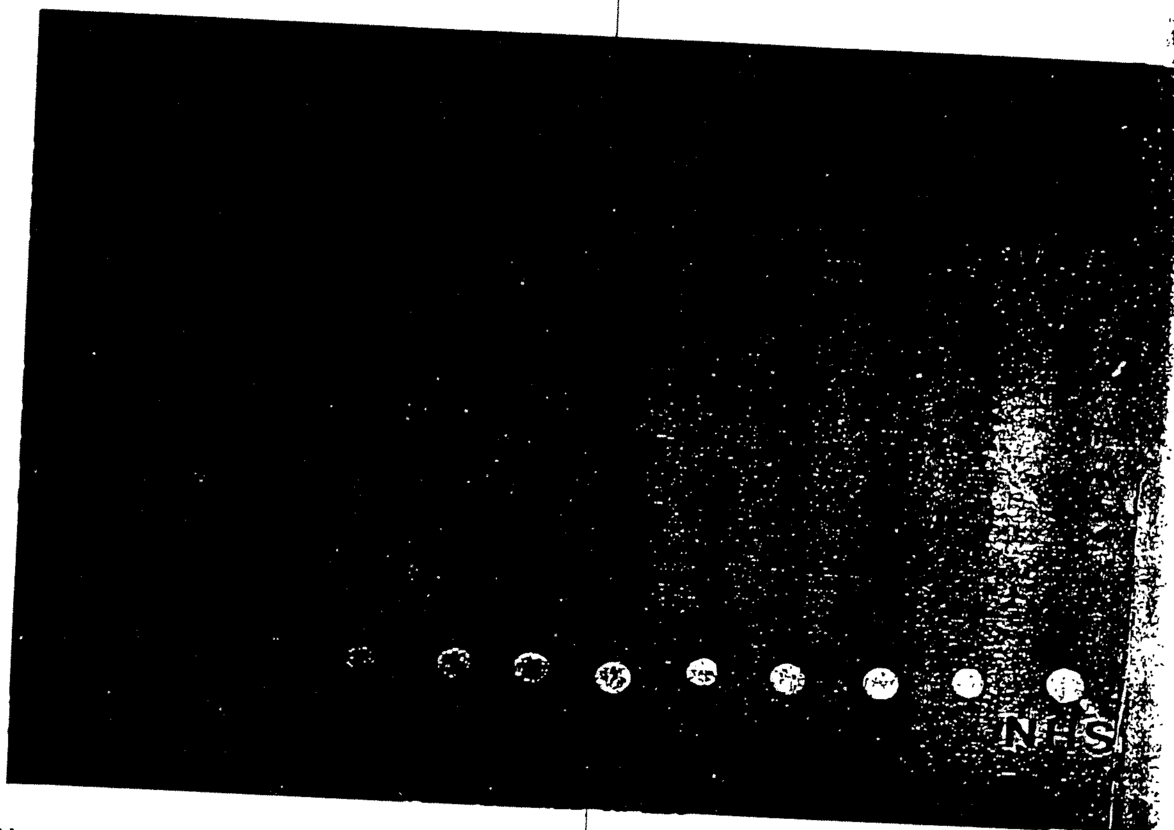

Figure 7. Rocket affinoelectrophoresis of 15 µl serum samples in a gel containing WGL (40 µg/ml agarose). Sample wells at both ends of the plate contained pooled normal human serum (PNHS) and the remainder of the wells contained the sera of anemic patients.

Table 2

ANEMIC DISORDERS

Patient	Rocket Height Patient/Control (mm)	Hematocrit (%)	Fetal Mouse Liver Cell Assay (mU Ep/ml)	Diagnosis
BB	32.5/29.5	21.0	20	Hemolytic Anemia
AM	30.5/29.0	24.9	220	Hemolytic Anemia
BS	28.0/28.0	20.0	20	Pernicious Anemia
CY	30.0/29.0	15.9	70	Iron Deficiency
WB	34.0/29.5	21.6	280	Refractory Anemia
BSm	33.5/27.0	28.4	50	Iron Deficiency

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Table 2 (Continued)

CANCER PATIENTS

Patient	Rocket Height Patient/Control (mm)	Hematocrit (%)	Fetal Mouse Liver Cell Assay (mU Ep/ml)	Diagnosis
VL	29.5/26.5	21.9	70	Ovarian Cancer
JS	29.5/28.5	24.9	100	Stomach Cancer
ED	36.5/26.0	25.2	280	Prostate Cancer
PH	28.5/26.0	20.7	60	Myeloproliferation
RM	30.5/27.0	26.1	90	Leukemia (AML)
LC	31.0/27.0	23.1	140	Lymphoma

When WGL-affinoelectrophoresis was repeated on serum samples from the same 30 anemic individuals, comparable correlation of SRH:NRH ratio with both hematocrit and Ep level were obtained. Reproducibility of rocket heights was excellent for the two experiments ($t = 11.138$, $p < 0.0005$). Correlation of SRH:NRH ratio with hematocrit was poor, probably because most of the serum samples came from cancer patients who often do not give a good negative correlation to Ep levels.

Since WGL-affinoelectrophoresis is a convenient method for ascertaining changes in the concentration of WGL-binding glycoprotein(s) in serum, we next investigated whether WGL could be used directly in a two-dimensional variant of rocket affinoelectrophoresis which we have termed "crossed affinoelectrophoresis" (by analogy with crossed immuno-electrophoresis). In Figures 8 and 9 are shown the potential of WGL to differentiate WGL-binding glycoprotein(s) in serum into three separate groups according to their electrophoretic mobilities. Both pooled normal human plasma (PNHP) and pooled normal human sera (PNHS) revealed similar patterns in crossed affinoelectrophoresis (Figure 8).

Clearly shown in Figure 9 is that in the sera of anemic patients, all three peaks are elevated compared to that of PNHP or PNHS, but the most anodal peak is significantly elevated. The Ep level in serum of anemic patients, represented by Figures 8A and 8B, was 290 and 1,130 mU Ep/ml respectively.

D. *Immunoradiometric Assay for Ep*

IgG antibodies were isolated for radioiodination from three different rabbit anti-Ep sera (R017, R94 and R1000). The CPM of radioactivity measured in each tube indicated that ^{125}I-rabbit IgG anti-Ep interacted with Ep adsorbed onto WGL-coated polystyrene balls in a linear relationship to an increasing concentration of Ep (Figure 4). Some of the sera derived from normal and anemic individuals were tested in this IRMA procedure.

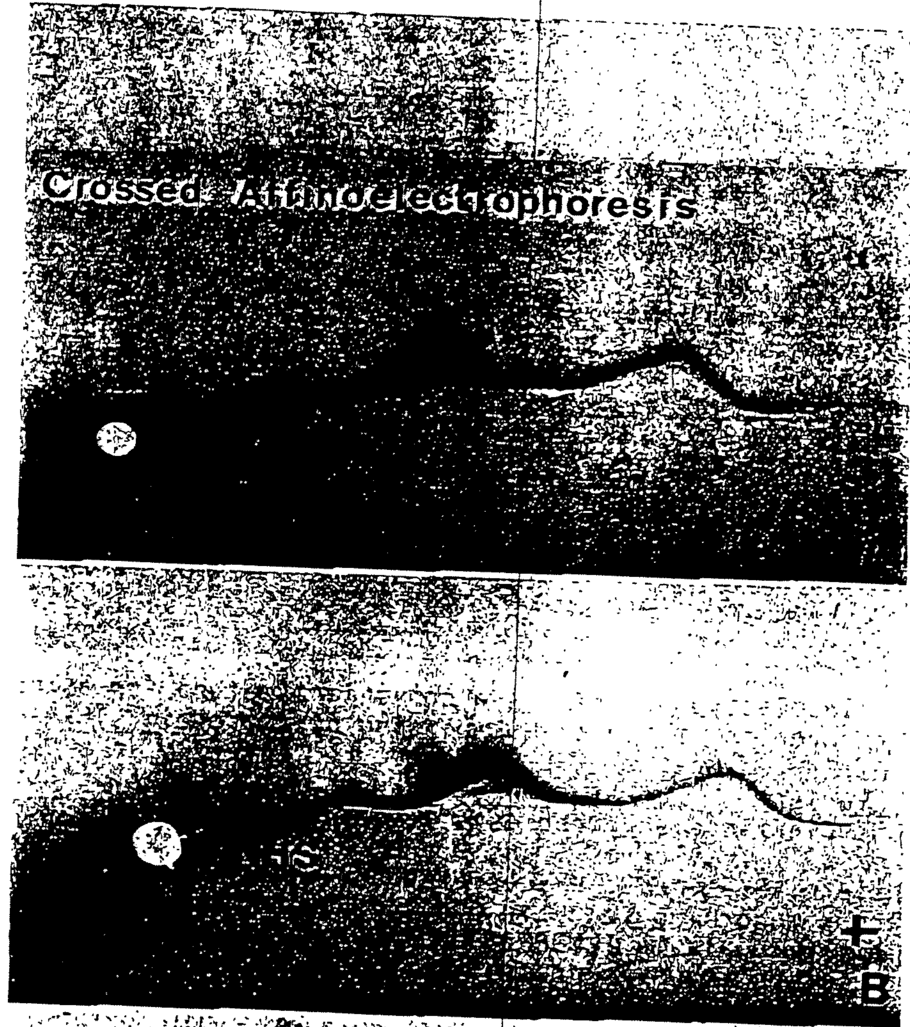

Figure 8. Crossed affinoelectrophoresis of 20 μl each of PNHP (8A) and PNHS (8B) in a gel containing WGL (60 μg/ml agarose).

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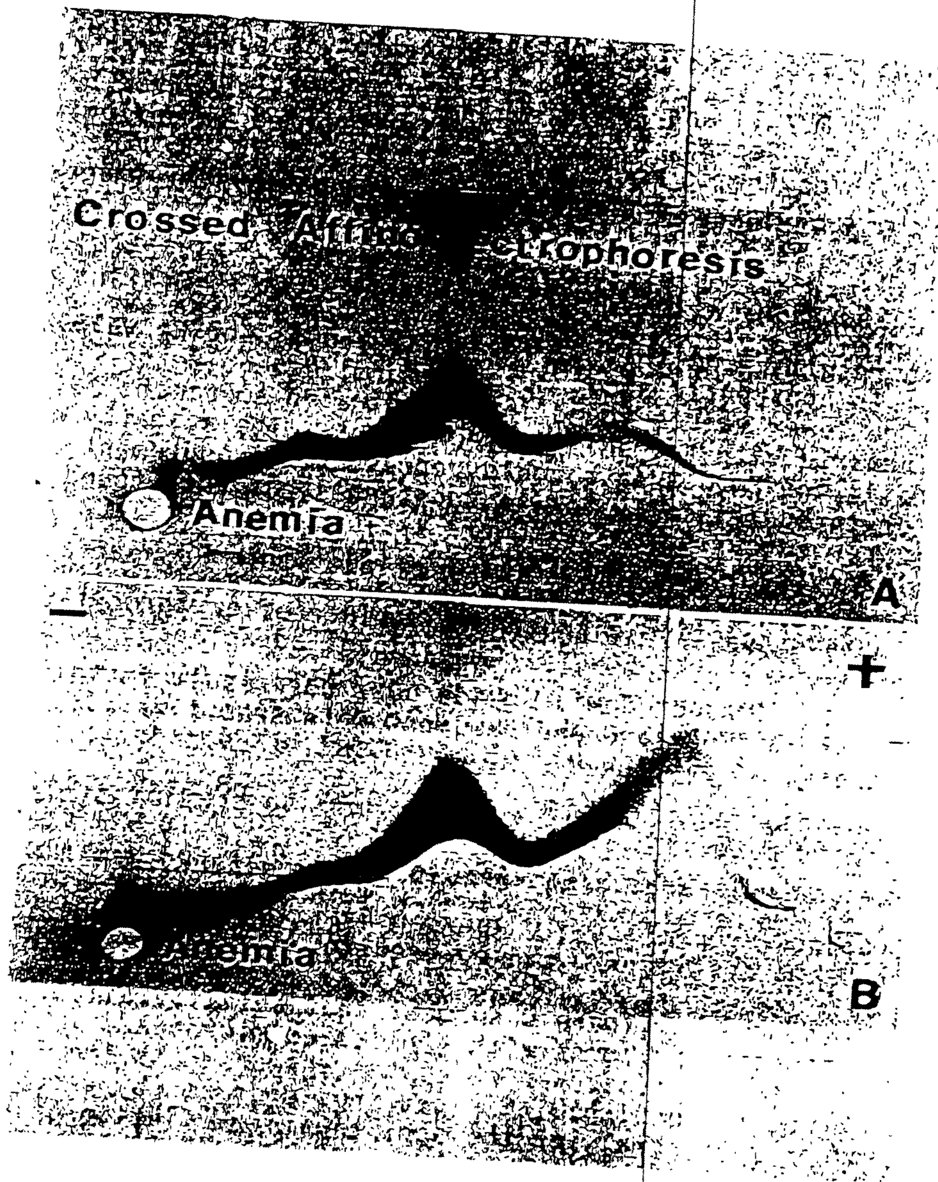

Figure 9. Crossed affinoelectrophoresis of 20 µl each of serum samples from anemic patients with elevated levels of Ep_{FMLC}.

DISCUSSION

When the figures for the levels of erythropoietin in normal human serum are compared, three facts emerge. First, when concentration methods are used lower values are often obtained. This is true for the erythrocythemic mouse assay used by Erslev and associates (40,41) (values <3.9 - 18 mU/ml) and the fetal mouse liver assay used by Napier (51) (10.4 mU/ml). Second, by the recently reported immunological assays and erythrocythemic mouse assays, levels of 20 - 35 mU/ml are found; i.e., HAI (30) (7 - 36 mU/ml), radioimmunoassays by three groups (5,35,36) (19 - 30 mU/ml), erythrocythemic mice (42,43) (25.8 and 34 mU/ml). Third, the recent values obtained by the FMLC assays are slightly higher (46,47,50)(29 - 50 mU/ml).

Based on the results from this variety of methods, the Ep value in normal serum is apparently about 30 ± 10 mU/ml depending on the method of assay. The lower values found in concentrated specimens may be due to losses incurred with the boiling techniques. The slightly higher values found in the FMLC assays could represent a measurement of additional erythropoietically active substances, as were found by Dunn et al. when concurrent assays were performed by the FMLC method and the erythrocythemic mouse assay (52).

The use of surface-reactive microbeads in place of erythrocytes as indicator particles offers several significant advantages:

1. Prolonged storage of microbeads does not result in hemolysis or contamination, and pretreatment with stabilizers is not necessary.
2. Microbeads provide a homogeneous, stable and standard indicator material.
3. Prior absorption of test sera is not necessary.
4. Microbeads are relatively inexpensive. A single vial of microbeads costing $70 can be used to assay 1,500 samples.

The results presented in Figure 5 suggest that serially diluted Ep produced agglutination patterns with sensitized microbeads identical to those obtained when sensitized erythrocytes were used as the indicator particles. Agglutination inhibition patterns produced by incubating an Ep standard or test sera with the agglutinating dilution of antiserum also appeared to produce results whether sensitized microbeads or erythrocytes were the indicator particles used. A direct comparison of the Ep titers obtained in a variety of sera using either sensitized erythrocytes or synthetic microbeads as indicator particles is currently under extensive investigation.

One problem with the immunochemical assay for Ep which is not overcome by the use of synthetic microbeads is the specificity of the assay. Thus, when "pure" Ep is not readily available, the monospecificity of the antisera used must remain in question. Nevertheless, the agglutination inhibition assay detects a serum factor which behaves as is

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expected of a humoral regulator of erythropoiesis (10,11). Furthermore, in our laboratory we found that the assay is not influenced by the wide variety of other hormones (e.g. ACTH, thyroxine, androgens, etc.) present in human serum (unpublished observations). Even though some difficulties with the commercially available HAI kit have been reported (53,54) under laboratory conditions, we find the assay useful; other investigators have found the technique to be reliable (30,55-57).

Ep_{WGL} Assay

The main advantage of Laurell quantitative immunoelectrophoresis is that it permits detailed analysis and quantitation of proteins even in crude mixtures (15). However, a prerequisite to its use is that the specific antibodies must be available to react with the antigen in question. The interaction of carbohydrates or glycoproteins with lectins appears to be similar to the interaction between antigens and antibodies, and lectins can be considered as "para-immunological tools" (58).

Wheat germ lectin is a glycoprotein capable of binding to two kinds of monosaccharides, viz. N-acetylneuraminic acid (sialic acid) and N-acetyl-D-glucosamine. Ep is also a glycoprotein, rich in carbohydrate (30%), which possesses terminal sialic acid residues in its carbohydrate side chains. Spivak et al. (59) and Rudzki et al. (60) have found that Ep activity is removed from Ep solution when it is passed through an agarose bound WGL column. The Ep activity can be recovered from the affinity column by eluting with N-acetyl-D-glucosamine. We felt that changes in the concentration of WGL-binding glycoproteins in the serum might reflect changes in the serum Ep concentration.

Affinoelectrophoresis or rocket affinity electrophoresis of the sera from normal and anemic individuals performed in an agarose gel containing WGL was used to quantitate glycoprotein(s) possessing N-acetyl-D-glucosamine and/or N-acetylneuraminic acid. WGL-affinoelectrophoresis, it was hoped, could be developed into a simple tool capable of assessing the anemic state of patients in conjunction with Ep in vitro bioassay. Preliminary results indicate that this technique should be investigated further.

It is clear from the rocket height of WGL-binding glycoproteins in the serum that the concentration of these proteins is much higher than that of bioactive Ep in the serum. Nevertheless, the change in Ep concentration is somehow reflected in the Ep level as determined by FMLC (a good correlation with $p < 0.025$). Further study is planned to identify and characterize these glycoproteins in the serum.

Ep_{IRMA}

In 1979, two reports (36,61) based on earlier observations (32,33) showed the feasibility of establishing a double antibody RIA for human Ep. Antisera were raised in rabbits with an impure Ep preparation and used without absorption. However, "pure" Ep (70,400 U/mg protein) was

radioiodinated with iodine-125 to serve as the ^{125}I-labeled antigen, and "pure" Ep was also used as a primary standard. Apparently, a parallel relationship was observed between the inhibition curves of Ep standard and the sera from anemic patients in competition against the antigen-antibody reaction of ^{125}I-Ep and anti-Ep antibody. Garcia et al. (36) and Sherwood and Goldwasser (61) reported the sensitivity of their Ep RIA to be 0.4 and 2-3 milliunits of Ep equivalent respectively. Rege and Fisher (5) have also reported the establishment of an improved radioimmunoassay. However, the double antibody RIA of Ep, albeit sensitive, will continue to present a problem. Since the half-life of ^{125}I-Ep is less than 60 days, "pure" Ep (which is in short supply) will need to be continuously iodinated.

We have selected a sensitive radioimmunological procedure called two-site immunoradiometric assay (IRMA) to measure the level of human Ep in serum or urine. In principle, this two-site IRMA involves the preliminary insolubilization of unknown antigen, e.g. Ep, by the first ligand-binding system (LBS), viz. WGL, which is attached to a solid-phase support such as the surface of a polystyrene ball. Essentially all of the Ep (either standard or unknowns) added to the polystyrene ball combined with the WGL, and then the ball was washed. The radio-labeled second LBS, i.e. ^{125}I-rabbit IgG anti-Ep, was then added to the system, with LBS^1-Ep-LBS^2 complexes being formed as a result. The labeled complex was insoluble and unreacted labeled antibody could be washed away. As the amount of Ep in either standard or unknown increased, the radioactivity in the solid phase increased (see Figure 4). This method has been termed "two-site IRMA" or "sandwich IRMA." Use of this technique is restricted to antigens which can bind simultaneously to at least two LBS.

The variations introduced in our two-site IRMA involves the novel idea of employing WGL and rabbit anti-Ep antibodies instead of two different anti-Ep antibodies in our LBS. The advantages of using WGL in the first LBS is two-fold: 1) Relatively pure and biospecific WGL can be prepared in good yield by affinity chromatography. 2) The specificity of WGL is directed toward sialic acid and/or N-acetyl-D-glucosamine residues of the carbohydrate side chains of Ep whereas the antigenic determinant of rabbit anti-Ep antibody very likely involves the protein core of Ep. As the result, two LBS will not likely interfere with each other in the binding of Ep.

So far, the values for Ep_{RIA} obtained by IRMA have been too high compared to those derived by FMLC. This result could be explained by the fact that a relatively "impure" Ep was used as a standard and the anti-Ep antibodies might not have been completely monospecific. However, because purified Ep is not readily available to be used by all investigators and because monospecific and monoclonal antibodies could possibly be produced by the new powerful lymphocyte hybridoma technique, the IRMA method of Ep assay should be pursued further.

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Ep Assays

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