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Quantitation of *E. coli* Protein Impurities in Recombinant Human Interferon-γ

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ABSTRACT

A multiple antigen ELISA for *E. coli* proteins (ECPs) that may be present in purified recombinant human interferon-γ (rIFN-γ) was developed. SDS-PAGE and Western blotting analyses showed that the assay antibodies reacted with a wide spectrum of ECPs in the standard and with ECPs in a production run. In spike recovery studies, rIFN-γ at concentrations of 0.05 mg/mL and higher augmented the immunoreactivity of the ECPs in the standard curve (1.3–40.0 ng ECPs/mL) by approx 50%. To determine ECP content in purified rIFN-γ, 0.2 mg/mL of rIFN-γ was added to the standard curve diluent to compensate for enhanced immunoreactivity. The assay was precise (interassay precision of ECP controls ≤4.1 %CV) and accurate with recoveries of 111–115% of expected for ECPs (15–40 ng/mL) spiked into purified rIFN-γ (1 mg/mL). Linearity of dilution for ECPs spiked into rIFN-γ was obtained ($r = 0.999$). Moreover, linearity of dilution was obtained for ECPs in "in-process" samples, demonstrating the required condition of antibody excess for this type of multiple antigen ELISA. ECPs were not detectable in several purified lots of rIFN-γ. Therefore, these lots contained <1.3 ppm ECPs.

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Index Entries: Interferon-gamma; impurity assays; *E. coli* proteins; protein interactions; recombinant proteins.

Abbreviations: ECP(s): *E. coli* protein(s); ELISA: enzyme linked immunosorbent assay; rIFN-γ: recombinant human gamma interferon; rGH: recombinant human growth hormone; BSA: bovine serum albumin; PBS: phosphate buffered saline.

INTRODUCTION

In order to determine the purity of recombinant DNA (rDNA)-derived products, process-specific immunoassays for host cell impurities have been developed for *E. coli* (*1–3*), yeast (*4*) and Chinese hamster ovary cell derived products (*5*). These assays are feasible with rDNA-based production methods since it is possible to obtain probable impurities free of product by performing the initial steps of the purification process using host cells that contain production plasmid but not the product gene (a blank run). Antibodies raised to the host proteins from such a blank run can be used in immunoassays, such as sandwich ELISAs, to complement HPLC methods and sensitive SDS-PAGE analyses in detecting protein impurities in final vial (*6,7*) and in process validation studies. These process-specific impurity assays can also provide powerful tools for development and troubleshooting of the manufacturing process.

During the course of validating an ELISA for ECPs in purified recombinant gamma interferon (rIFN-γ), it was found that the absorbance of the standard ECPs in combination with rIFN-γ preparations was much higher than the sum of their absorbances when assayed individually. Thus, the ECP concentrations determined in recovery studies were erroneously higher than expected. This report describes the development of the ELISA for ECPs, the rationale for modification of the assay buffer to include rIFN-γ, and the quantitation of ECPs in several purified lots of rIFN-γ.

MATERIALS AND METHODS

Production of rIFN-γ

Actimmune® recombinant human gamma interferon was manufactured at Genentech, Inc. (South San Francisco, CA).

Preparation of ECPs Specific to the rIFN-γ Process

A blank run was performed to obtain the most probable ECP impurities present in purified rIFN-γ. *E. coli* host cells containing the production plasmid but not the rIFN-γ gene were fermented at production

scale and ECPs were obtained by taking the cell paste through the initial stages of purification to the stage at which rIFN-γ would be approx 95% pure in a typical production run. This yielded the most probable protein impurities for this process (*1*). The protein concentration of ECPs and rIFN-γ was determined by the method of Lowry (*8*) using a bovine serum albumin standard. The ECP pool at this stage was then used as the standard for the ELISA and for immunization to produce specific antisera. It was also used to affinity-purity the resultant antisera.

Preparation of Antibodies Against ECP

Antibodies against the ECP pool were raised in rabbits according to established procedures. Each rabbit was immunized sc with 0.5 mg ECPs in complete Freund's adjuvant for the initial immunization. Subsequent injections (0.25 mg) were made every 3 wk in incomplete Freund's adjuvant. Sera were collected, pooled, and the immunoglobulin fraction was obtained according to the general method of Good et al. (*9*). Affinity-purified anti-ECP antibodies were obtained by purifying this immunoglobulin fraction on a column containing the ECP preparation coupled to CNBr-Activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden; coupling efficiency 98% by A_{280nm}). After washing the column with 0.01*M* phosphate buffered saline, pH 7.4 (PBS) containing additional 1*M* NaCl, the antibodies were eluted with 0.2*M* glycine-HCl, pH 2.4. The eluted fractions containing affinity-purified antibody were dialyzed against PBS and stored in aliquots at −70°C. Rabbit antibodies to rIFN-γ were prepared similarly.

Preparation of Enzyme-Antibody Conjugate

Horseradish peroxidase (HRP) (Boehringer Mannheim, Germany, Grade 1, Purity number ~3) was coupled to affinity-purified antibody by the sodium periodate method of Nakane and Kawaoi (*10*). Coupled enzyme-antibody conjugate was fractionated over a Fractogel TSK-HW 55F (EM Science, Gibbstown, NJ) column (2.5 cm×60 cm) using a PBS eluant. Fractions with 403 nm/275 nm absorbance ratios of 0.40–0.45 were pooled, aliquoted, and stored at −70°C.

SDS-PAGE Immunoblot Analyses

The standard ECPs, rIFN-γ, and rIFN-γ at several stages of purification ("in-process" samples) were analyzed by SDS-PAGE using 15% polyacrylamide gels as described by Laemmli (*11*). After electrophoresis, the gels were silver stained using the method of Oakley et al. (*12*).

Immunoblots were performed according to the method of Towbin (*13*). Briefly, proteins were transferred from the SDS-PAGE gels onto

nitrocellulose sheets using a transfer apparatus (Hoeffer Scientific Instruments, San Francisco, CA) at a current of 1 ampere for 40 min. Buffer containing 50 m*M* Tris, 5 m*M* EDTA, 150 m*M* NaCl, 0.05% Triton X-100, and 0.25% gelatin was used to block excess protein binding sites. The sheets were then incubated with HRP-conjugated antibody for 2 h. After washing, bound antibody was detected by exposing the sheets to substrate solution for 10 min as described by Adams (*14*).

ELISA Procedure

The ELISA was performed as follows: Polystyrene 96 well microtiter plates (Nunc-Immuno plate MaxiSorp F96, NUNC, Denmark) were coated overnight at 4°C with 100 μL/well of affinity-purified anti-ECPs (2.5 μg/mL) in carbonate buffer pH 9.6. The plates were washed in PBS containing 0.05% Tween 20 (PBS-Tween 20). Excess binding sites were blocked for 1 h with 200 μL/well of PBS-Tween 20 containing 0.1% gelatin, and 0.01% thimerosal (assay diluent). The plates were washed, and 100 μL of the standards, controls, samples were added in duplicate to the appropriate wells, and the plates were incubated for 2 h with agitation on a plate shaker (Mini-orbital shaker, Bellco, NJ). Calibration standards, controls and samples were initially diluted in assay diluent. Subsequently, for validation of the assay, they were diluted in assay diluent or assay diluent containing 0.2 mg rIFN-γ/mL. The plates were washed again and 100 μL of HRP-labeled anti-ECP antibodies in assay diluent were incubated in each well for 1 h with agitation. After another wash step, 100 μL of enzyme substrate solution (100 μL of 3% H_2O_2, 10 mg o-phenylenediamine, 25 mL of 50 m*M* sodium phosphate/25 m*M* citrate buffer, pH 5.0) was added to each well and the plates were incubated for 10–20 min in the dark at ambient temperature without agitation. The reaction was stopped by addition of 100 μL of 4.5*N* H_2SO_4 to each well. Absorbance was measured at 492 nm using a reference wavelength of 405 nm on a Titertek plate reader (Flow Labs, McLean, VA). The concentration of ECPs in samples was interpolated from the calibration curve obtained from ECP standards using a four-parameter curve fitting program written at Genentech based on the nonlinear least squares algorithm of Marquardt (*15*).

Calculation of ECP Content in rIFN-γ

Samples of ECPs were assayed as a dilution series in the ELISA assay to ensure that antibody excess was established (*1*). ECP concentration of the samples was interpolated from the standard curve in units of ng ECP/mL and ppm content was calculated as ng ECPs/mg rIFN-γ. Samples that yielded a constant ppm value (plateau) with dilution were presumed to be in antibody excess for the ECPs detected. Values obtained from serial dilutions that differed from one another by less than 20% were averaged and reported as the sample concentration.

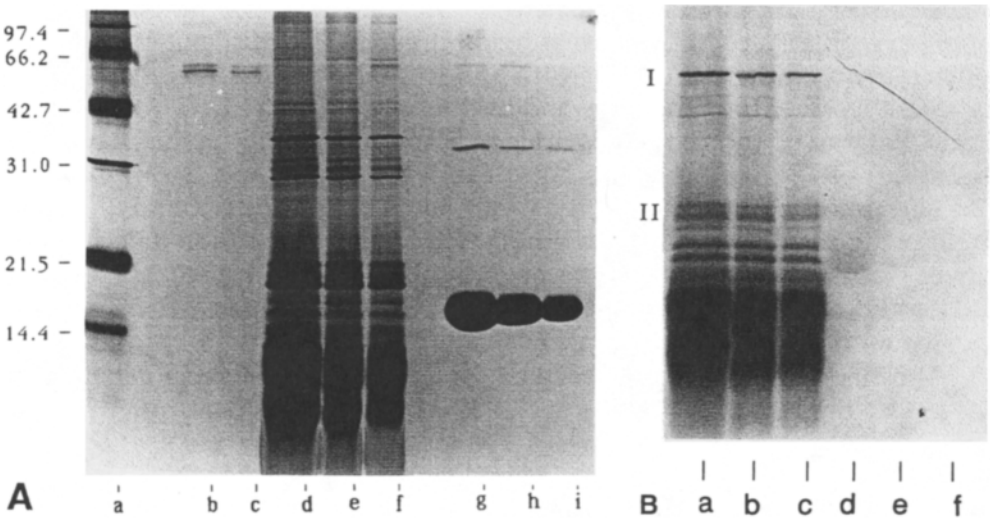

Fig. 1. SDS-PAGE Analysis of ECP Standard and rIFN-γ. *A. Silver Stain*—a: mol wt standards; phosphorylase b, 97.4 kDa; BSA, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa. b, c: 5 and 2 ng BSA, respectively. d, e, f: 20, 10, and 5 μg ECP standard, respectively. g, h, i: 20, 10, and 5 μg rIFN-γ. *B. Immunoblot using affinity-purified anti-ECP antibodies*—a, b, c: 20, 10, and 5 μg ECP standard, respectively; d, e, f: 20, 10 and 5 μg rIFN-γ, respectively.

Other Reagents

Bovine Serum albumin (BSA) and gelatin were obtained from Eastman Kodak (Rochester, NY). Protropin® human growth hormone (rGH) and human nerve growth factor were prepared at Genentech. Other chemicals were reagent grade or better.

RESULTS

Analysis of ECP Standard and Anti-ECP Antibodies

Figure 1A (lanes d–f) shows the SDS-PAGE analysis of the ECP standard obtained from the blank run preparation at different loads. Stained protein and peptide bands were visible over a wide range of molecular weights from greater than 97.4 kDa to less than 14.4 kDa. Antibodies to these ECPs were prepared in rabbits and affinity-purified as described in the Methods. The presence of antibodies to the individual bands were

demonstrated by immunoblotting a companion gel transferred to nitrocellulose (Fig. 1B, lanes a–c). Because of the wide range of molecular sizes and varying concentrations of individual bands it is difficult to visually determine if all the bands blot with anti-ECP antibodies. However, there is reactivity over the whole gel and as the load was increased from 5 to 20 μg ECPs, more bands became visible by immunoblotting. In fact, a careful scanning of the region above band I and between I and II of a freshly stained immunoblot shows the presence of additional bands. Thus, within the resolution of this method, the majority of bands detected by silver staining were also detected by immunoblotting. Hence, these anti-ECP antibodies should be able to detect ECP impurities which might co-purify with rIFN-γ.

Purified rIFN-γ, electrophoresed on the same SDS-PAGE gel at 5–20 μg loads, migrated with a mol wt of 17 kDa. A minor band migrated at about 34 kDa and trace amounts of a band migrated with a mol wt close to 66 kDa (Fig. 1A, lanes g–i). The 34 and 66 kDa bands probably represent nondissociable dimer and tetramer of rIFN-γ. These bands, when transferred to nitrocellulose, did not blot with anti-ECPs (Fig. 1B, lanes d–f) but did blot with anti-rIFN-γ antibodies (data not shown).

In order to estimate the purity of final vial rIFN-γ, 5 ng and 2 ng loads of bovine serum albumin were also electrophoresed on the same gel (Fig. 1A, lanes b,c). Since no peptide bands other than the three rIFN-γ bands mentioned above were detected by silver stained SDS-PAGE, the concentration of impurities for a single *E. coli* protein not comigrating with rIFN-γ, was estimated to be < 2 ng ECPs in a 20 μg sample of rIFN-γ (less than 100 ppm). For a more sensitive method of determining purity levels in rIFN-γ, the ELISA described below was developed.

Validation of ELISA for ECPs in rIFN-γ

Accuracy

Unlabeled and HRP-labeled affinity-purified anti-ECP antibodies were optimized for use in the double antibody sandwich ELISA to measure ECPs. The standard curve was generated with ECPs in the range 1.3–40 ng/mL. During initial spike recovery experiments to validate assay accuracy, it was found that the immunoreactivity of the ECP standard was increased by inclusion of rIFN-γ. This increase was not owing to ECP impurities in the rIFN-γ since rIFN-γ alone produced negligible absorbance in the assay (*see later*). The enhancement of ECP immunoreactivity was proportional to the rIFN-γ concentration and occurred throughout the assay range (Fig. 2). The initial effect was observed at 0.5 μg/mL and was maximal at concentrations of 200 μg rIFN-γ/mL (Fig. 2). On a second microtiter plate, 50, 100, and 200 μg rIFN-γ/mL resulted in identical curves (data not shown).

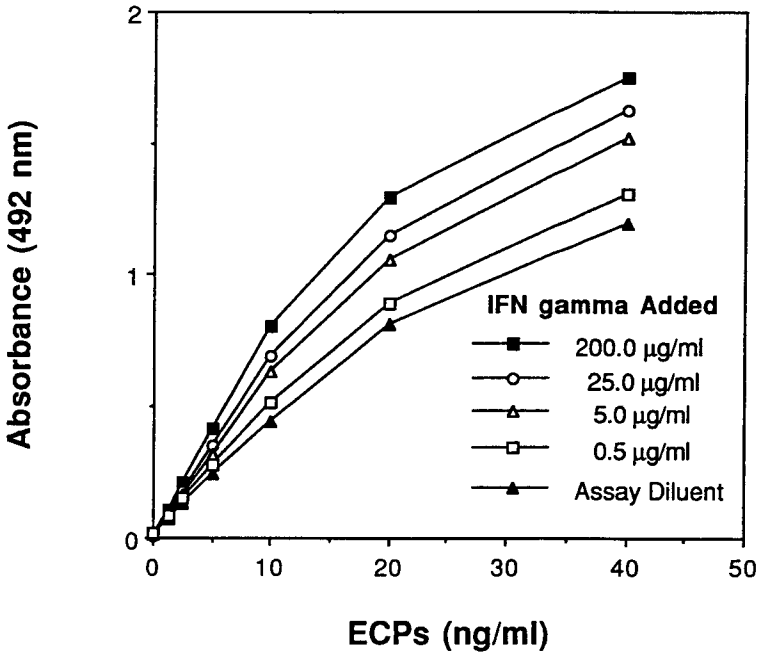

Fig. 2. Enhanced reactivity of ECP standards in presence of rIFN-γ. Twice the ECP standard concentration was mixed with an equal vol of rIFN-γ at twice the indicated concentration and assayed in the ECP ELISA format. Standards in ▲, assay diluent; □, 0.5 µg/mL rIFN-γ; △, 5.0 µg/mL; ○, 25.0 µg/mL; ■, 200 µg/mL. On a second microtiter plate, identical ELISA curves were obtained for ECP standards containing 50, 100, or 200 µg rIFN-γ/mL.

Three lots of rIFN-γ did not contain significant concentrations of ECPs as indicated by the negligible absorbance values obtained when these lots were assayed in the ELISA format without rIFN-γ in the assay diluent (Table 1). At 1 mg/mL the absorbance values ranged from 0.029 to 0.089, which were less than the absorbance value for the 1.3 ng/mL ECP standard.

In subsequent experiments, lot A was added to the assay diluent at a concentration of 0.2 mg/mL to compensate for the effects of rIFN-γ in assay samples. This concentration was chosen to ensure the maximum effect. Nevertheless, purified lots of rIFN-γ were tested subsequently at a concentration of 1 mg/mL in order to minimize the dilution of potential ECPs. Table 2 shows that acceptable spike recoveries of ECPs spiked into lots B and C were obtained (111–115%) when lot A was used as a component of the assay diluent.

Limit of Detection

Twenty replicates each of the buffer blank, 1.3 and 2.5 ng ECPs/mL were assayed to validate the lowest standard of the ELISA (Table 3). The

Table 1
Absence of Reactivity of rIFN-γ with anti-ECP Antibodies

Lot	rIFN-γ concentration mg/mL[a]	Absorbance at 492 nm[b]
A[c]	1.0	0.029
	0.2	0.017
B	1.0	0.044
	0.2	0.021
C	1.0	0.089
	0.2	0.029
Assay diluent[d]	0.0	0.019
1.3 ng/mL ECPs	0.0	0.105

[a]Protein concentration determined by absorbance at 280 nm using an extinction coefficient of 0.75.
[b]The reactivity of rIFN-γ preparations was compared with that of ECP standards diluted in assay diluent (without added rIFN-γ). Each value is the mean of duplicates.
[c]This lot of rIFN-γ was added to the assay diluent for subsequent validation of the ELISA for ECPs in rIFN-γ.
[d]Assay diluent without added rIFN-γ.

Table 2
Accuracy of the ECP ELISA[a]
Recovery of ECP Standard Added to rIFN-γ Lots

rIFN-γ Lot	ECPs added[b], ng/mL	Mean ECPs recovered[c]		Percent recovery[d] %
		Analyst 1, ng/mL	Analyst 2, ng/mL	
	0	<1.3	<1.3	—
B	15	17.0±10	17.6±0.5	115
	30	31.9±0.8	35.8±1.7	113
	40	42.9±2.5	48.0±1.6	114
	0	<1.3	<1.3	—
C	15	16.0±0.7	18.3±0.3	114
	30	31.1±1.3	35.4±1.7	111
	40	42.9±2.5	48.5±0.9	114

[a]The assay diluent contained rIFN-γ (0.2 mg/mL) of lot A.
[b]The concentration of ECPs added is the final concentration obtained in the sample after additions of a 0.66 mg/mL (determined by Lowry et al., 1951) ECP standard to 1 mg/mL preparations of rIFN-γ lots B and C.
[c]Each sample was assayed in duplicate by two analysts undiluted and as a twofold dilution series. Only those values that were within the range of the standard curve were used to calculate the mean ECP concentrations. Assay diluent contained 0.2 mg/mL rIFN-γ Lot A (Table 1).
[d]The percent recovery = (mean recovery)/added ng ECPs/mL) × 100.

Table 3
Sensitivity of the rIFN-γ ECP ELISA[a]

	Absorbance of 0 ng/mL ECP, buffer blank	Absorbance of 1.3 ng/mL ECP standard	Absorbance of 2.5 ng/mL ECP standard
N	20	20	20
Mean	0.082	0.161	0.253
SD	0.008	0.007	0.012
% CV	9.3	4.2	4.7
Mean+2SD	0.097		
Mean−2SD		0.147	0.229

[a]This method validates the lowest standard used in the assay as described by Anicetti et al. (*1*)

Table 4
Precision of the rIFN-γ ECP ELISA

	Low control	Middle control	High control
Intra-assay precision			
n	20	20	20
X, ng/mL	1.8	7.8	17.7
SD	0.04	0.08	0.54
% CV	2.0	1.0	3.0
Interassay precision			
n	12	12	12
X, ng/mL	1.8	8.0	18.7
SD	0.06	0.18	1.06
% CV	3.1	2.3	3.3

mean and standard deviation of the absorbance for the 1.3 ng ECPs/mL standard indicated that it was significantly different from the buffer blank. Thus, the 1.3 ng/mL standard was considered to be the limit of detection of this ELISA.

Precision

To test intraassay precision, twenty replicates of controls prepared from the ECP standard were assayed on the same plate. The concentrations of the controls were selected to cover the low, middle, and high portions of the standard curve (1.5, 7.5, 17.5 ng ECPs/mL). Percent coefficients of variation (%CV) were ≤ 3.0 (Table 4). The interassay precision of low, middle, and high controls was tested in twelve different assays. The %CVs were ≤ 3.3 (Table 4).

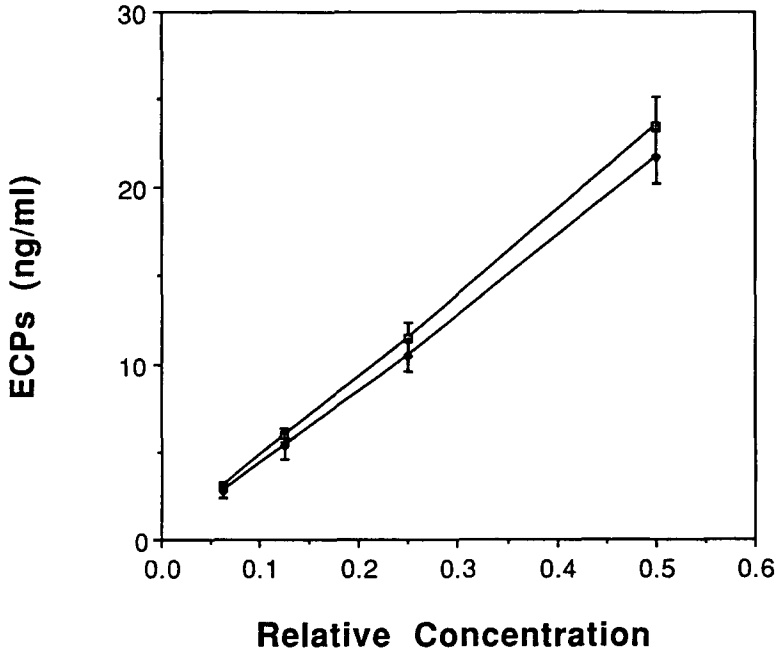

Fig. 3. Linearity of dilution of rIFN-γ ECP ELISA. Two rIFN-γ lots were spiked with 40 ng/mL ECPs and serially diluted with assay diluent containing 0.2 mg/mL rIFN-γ and assayed in the ELISA. The ordinate is the concentration of ECPs measured in the ELISA and the abscissa is the relative concentration (equal to the dilution factor) of ECPs in serial dilutions of the samples. For example, a 1:2 dilution of a sample has a relative concentration of 0.5. Values are the mean of two determinations each performed in duplicate.

Linearity

Linearity of dilution is particularly important in a multiple antigen immunoassay because it demonstrates the requisite condition of antibody excess (*1*). For this study, the ECP standard was used to spike two lots of rIFN-γ to a final concentration of 40 ng ECPs/mL. These samples were diluted serially twofold in assay diluent containing 0.2 mg rIFN-γ/mL. Figure 3 shows that ECP concentration determined in the ELISA was correlated linearly with dilution of these samples. (Correlation coefficient, *r*, was ≥ 0.999 for each of these samples).

Quantitation of ECPs in Purified rIFN-γ

Even though the blank run contains ECPs most likely to copurify with rIFN-γ, the possibility exists that additional ECPs may be present in production runs. Therefore, additional studies were performed to demonstrate that the assay antibodies could detect ECPs at various stages of the production process.

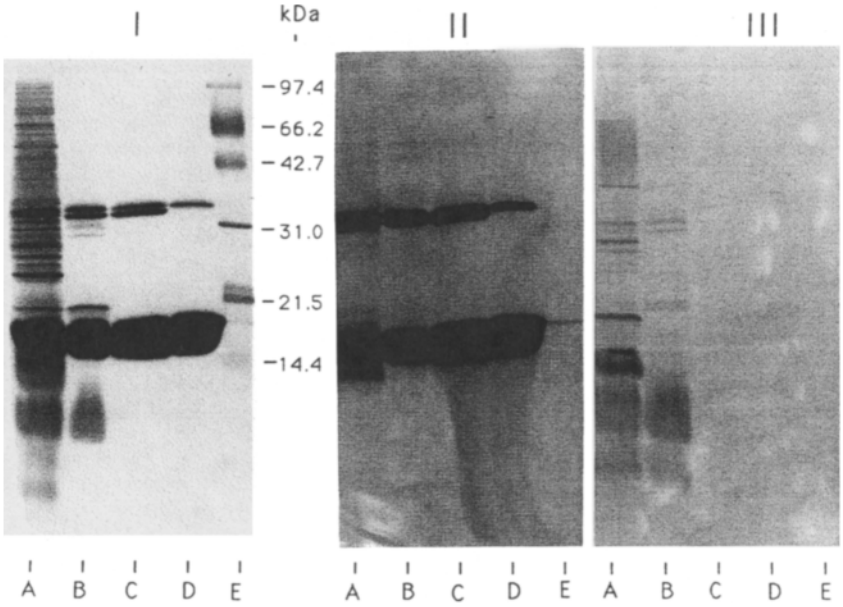

Fig. 4. SDS-PAGE Analysis of in-process rIFN-γ. I, silver stain; II, immunoblot with anti-rIFN-γ; and III, immunoblot with anti-ECP antibodies. A, B, C are consecutive steps in the purification process; D is a final vial rIFN-γ and E represents mol wt standards (*see* Fig. 1A). Samples containing 20 μg of rIFN-γ were loaded in lanes A, B, C, and D.

Figure 4, panel I shows the SDS-PAGE analysis of in-process samples and final vial rIFN-γ. Protein and peptide bands, stained with silver, covered a wide range of mol wt in the early stage of purification (Lane A). Fewer bands were detected in later stages of the purification process (lanes B, C), and in the final vial (lane D) only two major bands are visible. The faint band at approx 66 kDa seen in Fig. 1A is also visible on this gel. Figure 4, panels II and III show the peptide bands from companion gels after transfer to nitrocellulose and blotting with anti-rIFN-γ and affinity-purified anti-ECPs, respectively. The immunoblots detect bands which stain lightly in the silver stained gel (*see* panels I, II, III, lane B). The pattern of reactivity seen in Fig. 4, panel II and Fig. 4, panel III are different and demonstrate the presence of IFN-γ (and peptides containing IFN-γ determinants) and ECPs, respectively. In addition, the anti-ECP antibodies detect a wide spectrum of bands in the in-process rIFN-γ samples (panel III, lanes A and B). Lane A is taken at the same process step as the blank run ECP standard. Therefore, all of the bands should have been detected by the anti-ECP antibodies. Since one component (rIFN-γ) is present in far higher concentrations than the other components, it is difficult to load enough protein to detect minor bands and still see enough detail to determine whether ECPs comigrate with rIFN-γ. At higher protein loads, bands with mol wt greater than 66.2 kDa and between 21.5–31.0 kDa begin

Table 5
ECP Content of In-Process rIFN-γ and Purified Lots[a]

	Protein content, mg/mL[a]	ECP ELISA, ng/mL[b]	PPM
Step B	1.00	114,000	114,000
Step C	3.74	279	74.6
Step D[c]	17.80	24.9	1.4
rIFN-γ Lot B	1.00	LTS[d]	< 1.3
rIFN-γ Lot C	1.00	LTS	< 1.3

[a]Protein concentration determined by absorbance at 280 nm. An OD of 1.0 was taken to represent 1 mg/mL.
[b]Values for samples B, C, and D were calculated from the mean of at least two dilutions which yielded values on the standard curve.
[c]Different stage of purification process from that shown in Fig. 4.
[d]LTS. Less than lowest standard of 1.3 ng ECP/mL.

to be detected in immunoblots (data not shown). Thus, within the limitations of immunoblotting, it is concluded that antibodies are present which can detect ECPs during the production process. Furthermore, provided that each ECP has more than one epitope, the ELISA should be able to quantitate the ECPs.

When in-process samples were analyzed by the ECP ELISA, plateau values were obtained for steps B-D demonstrating that antibody excess had been achieved for the measured ECPs. As expected from the SDS-PAGE and Western Blot data, the ppm levels of ECPs decreased at later stages of the purification process (Table 5). No ECPs were detected in two rIFN-γ final vials. Since the sensitivity of the assay is 1.3 ng ECPs/mL and 1 mg rIFN-γ/mL samples were tested, the ECP levels were < 1.3 ppm.

Specificity

The specificity of the ELISA was examined by testing the reactivity of ECP preparations derived from purification processes for proteins other than rIFN-γ. ECP preparations specific to the processes for the purification of hGH, TNF-α and Tissue Factor were assayed in the rIFN-γ ECP ELISA (Table 6). The small degree of cross-reactivity (less than 0.31%) of ECPs obtained from other purification processes demonstrates that this assay is process-specific.

Effect of Various Proteins on Reactivity of IFN-γ ECPs in ECP ELISA

Since the pI of rIFN-γ is very basic (approx pH 10.5), it was possible that the enhanced reactivity of rIFN-γ ECPs was caused by the positive charge of rIFN-γ at neutral pH. Therefore, several basic and nonbasic proteins were tested to determine if they would also increase the reactivity of ECPs with the assay antibodies (Fig. 5). Inclusion of rIFN-γ, polylysine,

Table 6
Specificity of rIFN-γ ECP ELISA

Purification process[a]	ECP concentration, μg/mL	ECPs detected, ng/mL	Percent[b] cross reactivity
Human growth hormone	5	4.2	0.084
Tumor necrosis factor	100	2.0	0.002
Tumor necrosis factor	1000	4.4	0.0004
Tissue factor	1	3.1	0.31
Tissue factor	10	16.2	0.16

[a]ECPs from blank runs of purification processes of *E. coli* derived recombinant proteins.
[b]Percent cross reactivity = (ECP detected / Protein concentration) × 100.

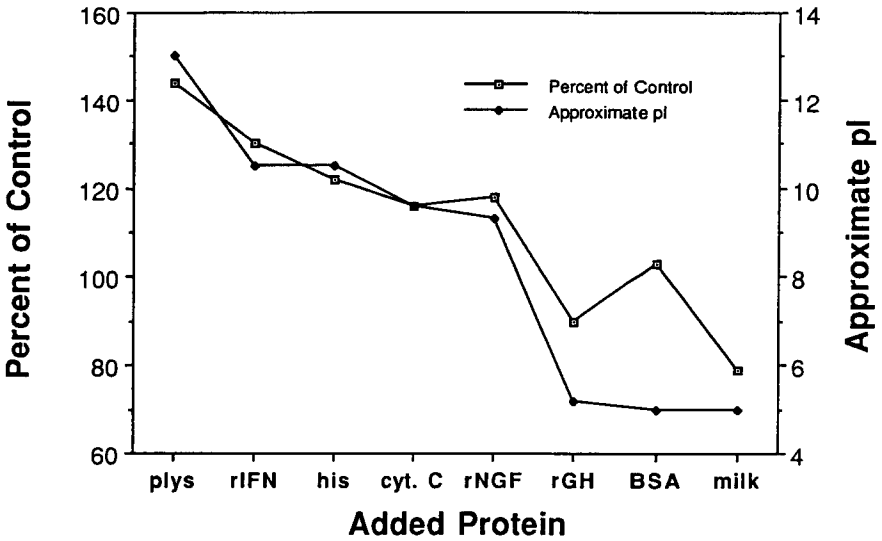

Fig. 5. Effect of various proteins on the reactivity of rIFN-γ ECPs. All proteins were added to assay diluent at a final concentration of 0.2 mg/mL except for nonfat dry milk, which was used at 0.1% (w/v). □; percent of control = [Absorbance (+)/Absorbance (−)] × 100, where Absorbance (+) = absorbance obtained in buffer containing the indicated protein and Absorbance (−) = absorbance obtained in the absence of the indicated protein. Values are the mean of duplicate determinations. The concentration of rIFN-γ ECPs was 40 ng/mL in all samples. ◆; approx pI as described in the text.

cytochrome C, histone, and nerve growth factor in assay diluent at a final concentration of 0.2 mg/mL increased the reactivity of ECPs in the ELISA (approx pIs 10.5, 13*, 9.6, 10–11, and 9.3, respectively). Neutral and acidic

*The theoretical titration curve for polylysine was calculated for a 50,000 dalton peptide, assuming no electrostatic interactions between the residues, using an intrinsic pK for the ε amino group of 10.4. Calculations were done using a computer program written by Steven J. Shire, Genentech, Inc.

Table 7
Effect of rIFN-γ on GH ECP ELISA*

GH ECPs, ng/mL	$A_{492\,nm}$ −IFN-γ	$A_{492\,nm}$ +IFN-γ
40	0.43	0.44
20	0.24	0.24
10	0.16	0.16
5	0.11	0.11
2.5	0.08	0.08

Growth hormone (GH) ECPs were assayed as a dilution series in either assay buffer or in 0.4 mg rIFN-γ/mL in the GH ECP ELISA. Absorbance values are the mean of duplicate determinations.

proteins (BSA, pI ≈ 5; rGH, pI ≈ 5.2; nonfat milk [≥ 90% casein], pI ≈ 4–6) had no effect or reduced reactivity of ECPs in the ELISA when added to assay diluent at a final concentration of 0.2 mg/mL (nonfat milk used at 0.1%, w/v). Protamine, a mixture of basic proteins, did not increase the reactivity of ECPs in the ELISA (data not shown). This lack of effect of protamine may be attributed to the lower mol wt of these peptides (4000–8000 daltons) compared with that of the other basic proteins tested (12000–70000 daltons).

Effect of rIFN-γ on rGH ECP ELISA

To verify that the effect of various proteins on the immunoreactivity of rIFN-γ ECPs was specific, ECPs from the rGH process were tested in the rGH ECP ELISA with and without 1 mg/mL of rIFN-γ. Table 7 shows that there was no effect over the range of 2.5 to 40 ng/mL ECPs. Similar to the rIFN-γ ECP ELISA, the rGH ECP ELISA also uses gelatin in the assay buffer to reduce nonspecific interactions. Thus, the effect of rIFN-γ on quantitation of ECPs was specific for the rIFN-γ, but not the rGH, process.

DISCUSSION

The immunoassay described above follows the procedures outlined in an earlier manuscript for quantitation of host cell impurities in recombinant DNA-derived proteins (*1*). By SDS and Western Blot methods, the assay antibodies demonstrated a broad spectrum of reactivity with blank run proteins and with in-process ECPs (Figs. 1 and 4). In addition, plateau values were obtained in the ECP ELISA for in-process samples. Therefore, this assay is valid for detection of potential ECPs in all stages of the rIFN-γ recovery process.

In the ELISA described here, rIFN-γ increased the signal obtained from the reaction between ECPs and immunoglobulins directed against

these ECPs. The effect of rIFN-γ was mimicked by inclusion of other basic proteins, but not by neutral or acidic proteins, in the reaction mixture. The diluent used throughout these studies contained gelatin, which carries a net negative charge at neutral pH (pI 4.7–5.0), to block nonspecific interactions of the reactants. The antibodies used in this multiple antigen ELISA are raised against blank run antigens that copurify with product through several stages of the purification process. Thus, process-specific ECPs share many of the physical properties of the product. The ECPs specific to the rIFN-γ process are basic proteins with isoelectric points in the range 9–11, whereas those specific for the rGH process are in the range 5–8 (unpublished data). It is possible that acidic proteins such as gelatin bind to ECPs specific to the rIFN-γ process and thereby interfere with binding of antibodies to the ECPs. Basic proteins may prevent binding of gelatin to the ECPs and, as a consequence, enhance the reaction between ECPs and antibodies. Similar effects of basic proteins were not observed in a gelatin-based ELISA for ECPs specific for the Protropin rGH process. This suggests that gelatin should be carefully evaluated as a carrier protein in impurity assays for basic ECPs.

A similar interference was observed in an assay for quantitation of potential Protein A impurity in a murine IgG product (*16*). In the development of that assay, it was found that murine IgG reduced the reactivity of the analyte, Protein A, with F(ab)'2 fragments of antibodies against Protein A. Thus, the reaction between the product and impurities interfered with immunological quantitation of the latter. The interference of product in the ELISA was maintained constant by inclusion of a uniform final concentration of murine IgG (purified by means other than Protein A) in all samples and standards. In contrast, the interference of an assay reagent (gelatin) in the rIFN-γ ECP ELISA described here has been reversed by inclusion of a uniform concentration of rIFN-γ in the reaction mixture. The lot of rIFN-γ chosen did not contain detectable concentrations of ECPs.

In agreement with earlier studies (*1*), we have found that assays for potential impurities in protein pharmaceuticals produced in recombinant organisms are process-specific and require careful validation. Moreover, components of assay reagents may interfere with the quantitation of impurities and high concentrations of product may neutralize such interference. When carefully validated, multiple antigen ELISAs can provide accurate and precise quantitation of potential impurities at ppm levels.

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