An Enzyme-Linked Immunosorbent Assay (ELISA) for Quantitation of Adducts of Granulocyte–Macrophage Colony Stimulating Factor (GM-CSF) and Human Serum Albumin (HSA) in Stressed Solution Mixtures

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HPLC analyses of GM-CSF in solution mixtures containing both GM-CSF and HSA showed losses of GM-CSF which could not be accounted for using conventional electrophoretic and/or RP-HPLC techniques. Further investigation of these mixtures by immunoblotting and by immunoaffinity chromatography demonstrated the presence of high molecular weight (>67,000) GM-CSF related species. No such species was detectable in solutions of GM-CSF alone. This experiment pointed to the formation of an adduct between GM-CSF and HSA in the solution mixtures. To probe further the hypothesis of a GM-CSF/HSA adduct, an immunologically based test was conceived which could react only with this type of hybrid molecule. A sandwich enzyme-linked immunosorbent assay (ELISA) was developed using two antibodies, anti-GM-CSF (capture antibody) and anti-HSA (detection antibody), as part of the quantitation of GM-CSF/HSA adducts. After confirming its existence by ELISA, a GM-CSF/HSA adduct was isolated from the solution mixture containing both GM-CSF and HSA. This isolate served as a primary reference standard in the ELISA assay. The immunoassay has a subnanogram sensitivity and is highly specific for GM-CSF/HSA adducts in the presence of either free GM-CSF or free HSA. As a verification, conjugates of GM-CSF/HSA were synthesized using a cross-linking reagent. These covalent conjugates reacted positively in the ELISA and are employed as a convenient alternative reference standard.

KEY WORDS: granulocyte–macrophage colony stimulating factor (GM-CSF); human serum albumin (HSA); GM-CSF/HSA adducts; enzyme-linked immunosorbent assay (ELISA); thermal degradates.

INTRODUCTION

Human granulocyte–macrophage colony stimulating factor (hGM-CSF) is a clinically useful therapeutic protein (1–3). Recombinant hGM-CSF produced in *Escherichia coli* has a molecular weight of 14.6 kD and is a single-subunit protein (4,5). To maintain biopotency, recombinant-derived therapeutic proteins are often formulated with stabilizers (6,7). Human serum albumin (HSA) is a candidate excipient to stabilize rhGM-CSF. The use of HSA has stemmed from its good solubility, thermal stability, and ability to prevent surface adsorption of active protein drugs (6,7). Protein nucleophiles such as the ε-amino group of lysine, cysteine sulfhydryl, and tyrosine hydroxyl groups in HSA have been implicated in the binding of a wide variety of drugs such as diazepam, warfarin, and digitoxin (8). In this report we studied the covalent bonding of GM-CSF with HSA in solution mixture and developed a sensitive enzyme-linked immunosorbent assay (ELISA) for quantitation of GM-CSF/HSA adducts in solution mixtures.

MATERIALS AND METHODS

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-Page) and Immunoblotting of GM-CSF/HSA Mixtures

A lyophilized mixture of GM-CSF (recombinant version isolated from genetically engineered *E. coli* at Schering Corporation, Kenilworth, NJ) and HSA (Armour Co., IL) was reconstituted with 1 mL of Milli-Q water and the solution mixture was used in all the studies. Unless indicated otherwise, the solution mixture contained 50 μg/mL and 1 mg/mL of GM-CSF and HSA, respectively. HSA at 1 mg/mL in water containing stabilizers was employed as a control. Aliquots of the GM-CSF/HSA solution mixture and the control were analyzed by electrophoresis on 14% polyacrylamide slab gels under reducing and nonreducing conditions using the Laemmli system (9). Proteins in gels were detected by silver staining (10). For immunoblotting, proteins separated by SDS-PAGE were electrophoretically transferred to a nitrocellulose membrane (11) using Tris–glycine buffer containing 20% (v/v) methanol (12). After blocking the membrane with 2% (w/v) gelatin, the blots were incubated overnight with rabbit anti-GM-CSF serum in Tris-buffered saline (TBS) containing Tween-20. Immunoreactive protein bands were visualized by incubating the blots with the peroxidase coupled goat anti-rabbit IgG (Kirkegard & Perry Labs, MD; 1:500 dilution in TBS) followed by 4-chloro-1-naphthol/H_2O_2 substrate solution. Colloidal gold stain was used for direct visualization of proteins transferred onto the nitrocellulose membrane (13).

Affinity Purification of Polyclonal Antibodies Against GM-CSF and HSA

Polyclonal antibodies against purified GM-CSF were produced in rabbits by the standard immunization with purified GM-CSF in complete Freund's adjuvant. Antibodies were purified by affinity chromatography of pooled antiserum on a column of GM-CSF covalently linked to Sepharose 4B. After washing the column with phosphate-buffered saline (PBS; 10 m*M* sodium phosphate, pH 7.4, and 150 m*M* NaCl), the bound antibodies were eluted with 0.1 *M* citric acid/0.3 *M* NaCl and dialyzed extensively against several changes of PBS. Polyclonal antibodies against HSA were raised in goat by immunization with complete Freund's adjuvant and purified by affinity chromatography of pooled goat antiserum on a column of HSA covalently linked to

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Sephrose 4B. Affinity-purified goat anti-HSA antibodies were conjugated with horseradish peroxidase by the periodate oxidation method of Nakane and Kawaoi (14). The molar enzyme/antibody protein ratio was kept at 4 during the conjugation.

Preparation of GM-CSF/HSA Adducts for Use as a Reference Standard

A solution mixture containing both GM-CSF and HSA (50 μg/mL and 1 mg/mL, respectively) was filtered using an Ultrafree-MC filter unit (30,000 cutoff membrane from Millipore Corporation, MA; Catalog No. UFC3TTK00) to remove free GM-CSF. The retentate was washed twice with 400 μL of PBS. Proteins retained on the membrane were taken up with 200 μL of PBS. The retentate was then analyzed by SDS-PAGE and the amount of GM-CSF released from adducts following reduction with 2-mercaptoethanol was estimated visually by silver staining of the gel in comparison with known amounts of GM-CSF run in parallel. The retentate containing large amounts of HSA and also the GM-CSF/HSA adducts were further characterized for use as a standard in subsequent ELISA (see Results and Discussion).

ELISA for GM-CSF/HSA Adducts

Microtiter wells (Immulon plates from Dynatech Labs, VA) were coated overnight at 4°C with 100 μL of affinity-purified rabbit anti-GM-CSF antibody (5 μg/mL) in 50 m*M* carbonate buffer, pH 9.5. Unbound antibody was removed by washing wells with PBS + Tween-20, followed by incubation with samples diluted with PBS-Tween-20 for 3 hr at room temperature. Plates were then washed and 100 μL of affinity-purified goat anti-HSA antibody conjugated with peroxidase was added to each well and incubated for 2 hr at room temperature. After washing unbound anti-HSA peroxidase, a substrate solution (0.1% *O*-phenylenediamine + 0.015% H_2O_2 in phosphate/citrate buffer) was added. The resulting color development was terminated by the addition of an equal volume of 4 *N* H_2SO_4 after a few minutes, and the absorbance at 492 nm was measured on an ELISA plate reader. A standard curve was prepared using the reference standard mentioned above.

Immunoaffinity Purification of GM-CSF/HSA Adducts

Rat monoclonal antibody (2F10) produced against the first 16 amino acids containing peptide of GM-CSF was kindly provided by Drs. J. Raman and J. Mesuk of the Biotechnology Division of Schering-Plough Research. The IgG fraction of this monoclonal antibody was further purified by affinity chromatography on Gammabind G-agarose (Genex Corporation, MD). Approximately 4 mg of purified IgG was covalently coupled to 1 mL of Affi-Gel 10 (Bio-Rad, CA) according to manufacturer's instruction. Aqueous solution preparations of GM-CSF/HSA (50 μg/mL of GM-CSF and 1 mg/mL of HSA) and HSA (1 mg/mL) alone were thermally stressed at 55°C for 24 hr. The solutions were then separately filtered using the Ultrafree-MC filter unit (MW cutoff, 30,000). The retentates obtained were chromatographed in parallel on columns of monoclonal anti-GM-CSF-Affi-Gel 10. After washing columns extensively with PBS, the bound protein was eluted with 0.1 *M* citric acid/0.3 *M* NaCl, pH 2.5, dialyzed against PBS, and stored at −20°C until use.

Synthesis of GM-CSF/HSA Conjugates

N-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pierce, IL), a heterobifunctional reagent (15), was used to cross-link GM-CSF via cysteine to HSA (Sigma Co., MO). HSA, 150 nmol in 0.5 mL of PBS, was reacted with 2 μmol of SPDP dissolved in ethanol for 30 min at room temperature. Unreacted SPDP was removed by gel filtration on a column of Sephadex G-25, then 70 nmol of GM-CSF was reduced with 160 m*M* 2-mercaptoethanol in a buffer containing 0.1 *M* Tris–HCl, pH 8.0, 1 m*M* EDTA, and 4 *M* guanidine hydrochloride for 60 min at room temperature. The reduced GM-CSF was recovered by batch chromatography on a Sep-Pak C18 cartridge (Waters Associates, MA) with 90% acetonitrile/0.1% TFA and dried in a speed-vac. The reduced GM-CSF was then dissolved in PBS containing 4 *M* guanidine hydrochloride, mixed with 15 nmol of SPDP-derivatized HSA, and incubated overnight at room temperature. The coupling of GM-CSF to HSA was monitored by measuring the formation of pyridine-2-thione at 342 nm and also by SDS-PAGE under reducing/nonreducing conditions.

RESULTS AND DISCUSSION

When solution mixtures containing different ratios of GM-CSF and HSA were analyzed by SDS-PAGE (nonreducing conditions) followed by immunoblotting with rabbit anti-GM-CSF serum, several high molecular weight (>67,000) immunoreactive species were detected (Fig. 1). The absence of such species in solutions of either GM-CSF or HSA alone (Fig. 1, Lanes B and C) suggested that these species might have arisen from the interaction of GM-CSF with HSA in the mixture. The immunoreactive species were heterogeneous and their intensity varied with the ratio of GM-CSF and HSA in the solution mixtures (Figure 1, Lanes A, D, E, and F). Differences were also seen in solution mixtures with identical ratios of GM-CSF and HSA (Fig. 1, Lanes D and E). Solution mixtures aged at 4°C for 1 year showed additional immunoreactive species running below HSA and an oxidized species of GM-CSF running above GM-CSF band (Fig. 1, Lane F). To ascertain whether GM-CSF could be released from high molecular weight species, the solution mixture (containing GM-CSF and HSA at a ratio of 1:20) was freed of monomeric GM-CSF by membrane filtration (molecular weight cutoff, 30,000). The retentate was analyzed by SDS-PAGE under nonreducing/reducing conditions (Fig. 2). The release of GM-CSF following reduction was readily detectable by silver staining of the gel (Fig. 2, Lanes 1 and 2). The gel was deliberately overloaded to demonstrate the effectiveness of membrane filtration to remove GM-CSF and also to increase the detectability of GM-CSF following reduction with mercaptoethanol (Fig. 2).

We then investigated the effect of temperature on the formation of high molecular weight GM-CSF species in so-

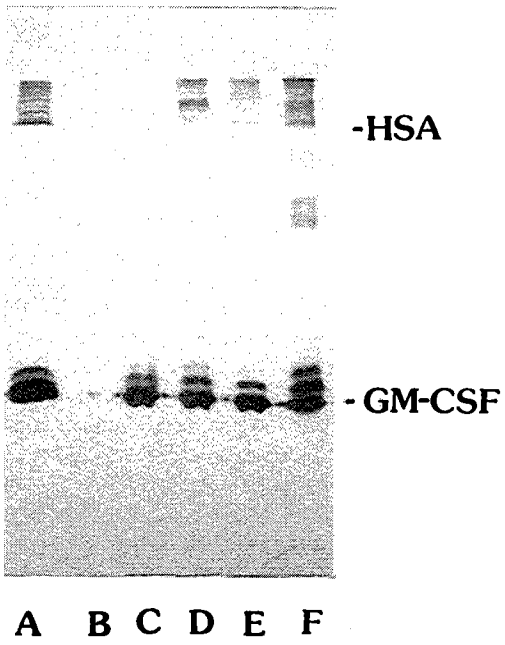

Fig. 1. Analysis of GM-CSF/HSA solution mixtures by SDS-PAGE/immunoblotting. Approximately 2 μg of GM-CSF from each solution mixtures containing differing amounts of GM-CSF and HSA was subjected to SDS-PAGE (14% gel) under nonreducing conditions. Proteins were then electrophoretically transferred onto a nitrocellulose membrane and immunoblotted with rabbit anti-GM-CSF antibody as described under Materials and Methods. Samples were from different solution mixtures of GM-CSF/HSA at the following ratios of GM-CSF to HSA: Lane A, 0.05:1.0; B, HSA alone; C, GM-CSF alone; D, 0.15:1.0; E, 0.15:1.0; F, 0.5:1.0 (aged). Migration of standard HSA and GM-CSF is indicated at the right.

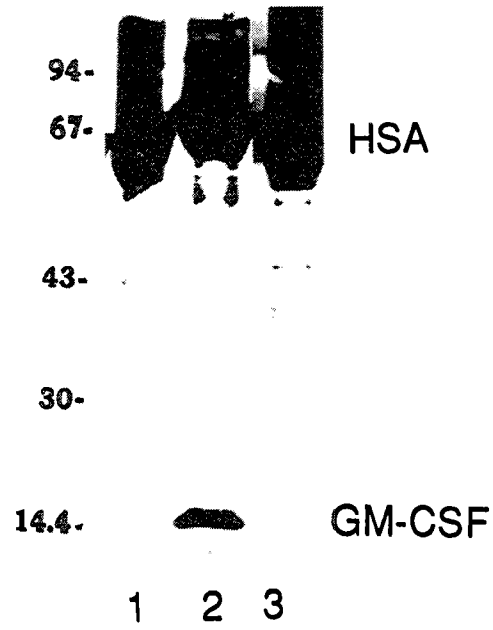

Fig. 2. Release of GM-CSF from adducts by 2-mercaptoethanol. Solution mixtures of GM-CSF/HSA and HSA alone were thermally stressed at 55°C for 2 days and filtered by membrane filtration. The proteins in the retentates (15 μg each) were separated by SDS-PAGE under nonreducing and reducing conditions and detected by silver saining as described under Materials and Methods. Lane 1, retentates of GM-CSF/HSA solution mixtures under nonreducing conditions; Lane 2, retentates of GM-CSF/HSA solution mixtures under reducing conditions; Lane 3, retentates of controls (HSA only) under reducing conditions.

lution mixtures. Figure 3 shows the immunoblotting profile of the retentates of solution mixtures incubated at different temperatures. Both reducible and nonreducible GM-CSF species were present in solution mixtures at 4, 25, and 37°C and were dramatically increased at 55°C (Fig. 3). There were significant amounts of GM-CSF-reactive species running below 67,000, probably representing breakdown products from high molecular weight species and/or nonreducible aggregates of GM-CSF (Fig. 3).

Since thermal stressing of proteins is known to result in a wide variety of physical and chemical changes in the molecule (16,17), we performed immunoaffinity chromatography of stressed solution mixtures to find out how many of the immunoreactive high molecular weight species were GM-CSF/HSA adducts. Solution mixtures containing both GM-CSF and HSA and HSA alone were stressed at 55°C for 24 hr. Following membrane filtration to remove monomeric GM-CSF, the GM-CSF/HSA mixture and HSA control were chromatographed in parallel on columns of monoclonal anti-GM-CSF antibody covalently linked to Affi-Gel 10. Approximately 2–6% of the applied proteins from the GM-CSF/HSA samples was immunologically bound to the column and eluted with citric acid, pH 2.5. No detectable amount of protein from the HSA solution control was eluted from the column, showing that there was no nonspecific binding of HSA to the affinity column. SDS-PAGE analysis of the column fractions under nonreducing conditions showed diffuse bands running below 67 kD and above 94 kD and a significant amount remained near the top of the gel (Fig. 4, Lane B). The reason the immunoaffinity-purified adducts from stressed solution mixtures migrated faster than 67 kD under

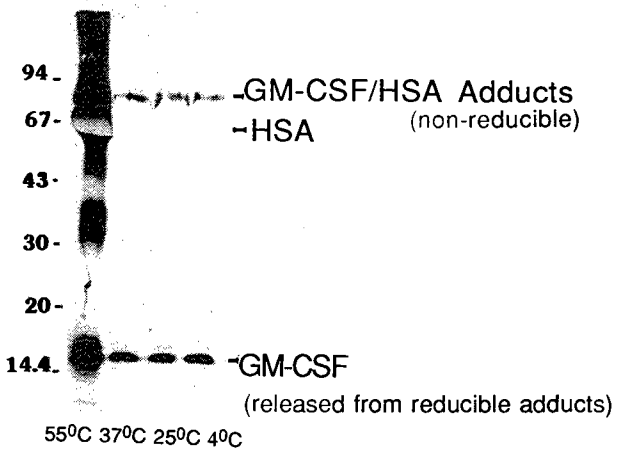

Fig. 3. Immunoblotting of thermally stressed solution mixtures of GM-CSF/HSA. Solution mixtures of GM-CSF/HSA were thermally stressed at different temperatures for 2 days and filtered to remove monomeric GM-CSF. The proteins (60 μg each) in the retentate were separated by SDS-PAGE under reducing conditions and immunoblotted with anti-GM-CSF antibody as described under Materials and Methods.

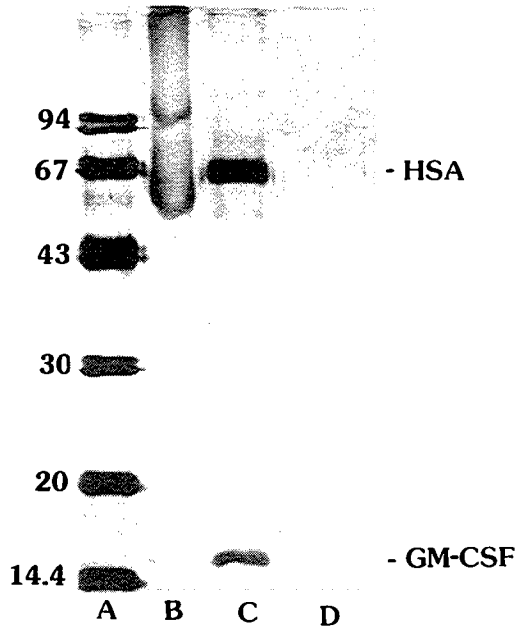

Fig. 4. Isolation of GM-CSF/HSA adducts by immunoaffinity chromatography. Proteins in the reconstituted, thermally stressed GM-CSF/HSA solution mixture and control (HSA alone) were subjected to immunoaffinity chromatography on columns of monoclonal anti-GM-CSF IgG covalently linked to Affi-Gel 10 as described under Materials and Methods. Protein fractions eluted from the column were analyzed by SDS-PAGE followed by silver staining. Lane A, protein molecular weight markers in decreasing order of molecular weight from the top of the gel: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and β-lactalbumin (14,400). Lane B, proteins eluted from GM-CSF/HSA sample electrophoresed under nonreducing conditions. Lane C, same as Lane B but treated with 2-mercaptoethanol. Lane D, fraction eluted from the control sample (HSA alone) treated with 2-mercaptoethanol.

nonreducing conditions is now known. However, it has been consistently noted by us that the standard HSA under nonreducing conditions migrated faster than the reduced HSA in SDS-PAGE. The majority of the affinity-purified adducts appeared to be disulfide bonded (Fig. 4, Lanes B and C). No such protein species were detected when the HSA solution control was subjected to immunoaffinity chromatography and electrophoresis (Fig. 4, Lane D).

Thus it was concluded that the formation of a GM-CSF/HSA adduct was one of the potential degradation products when a solution mixture containing both GM-CSF and HSA was subjected to thermal stress. The need for a sensitive method for their detection and quantitation was apparent. In reverse-phase HPLC (C-4 column; TFA/acetonitrile system), the adduct gave a broad elution profile. HPLC was not sensitive enough to detect submicrogram quantities of adducts. Enzyme linked immunoassays are extensively used for sensitive and rapid quantitation of analytes (18,19) and are increasingly used to analyze the purity of recombinant-derived protein pharmaceuticals (20,21). So we developed a sandwich or two-site enzyme immunoassay for detection of GM-CSF/HSA adducts. The affinity-purified anti-GM-CSF antibody adsorbed onto a titer well was used as the capture antibody, and the anti-HSA antibody conjugated to peroxidase was used as the detector antibody (Fig. 5).

Designation of a Reference Standard for ELISA

A solution preparation of GM-CSF containing HSA (50 μg/mL GM-CSF and 1 mg/mL HSA) whose immunoblotting profile showed a significant amount of GM-CSF/HSA adducts was selected as a source of reference standard for the ELISA. Free GM-CSF in this mixture was removed by membrane filtration (30,000 molecular weight cutoff) since it interfered with the assay by competing for the capture antibody. The retentate containing GM-CSF/HSA adducts and large amounts of HSA was analyzed by SDS-PAGE and immunoblotted under reducing and nonreducing conditions. The amount of GM-CSF released following reduction with mercaptoethanol was assigned by silver staining and comparison of the silver stain intensity to known amounts of GM-CSF run in parallel. Thus, the reference standard defines a concentration of adducts in terms of mercaptoethanol-releasable GM-CSF.

Specificity of the ELISA

The ELISA system described is highly specific for GM-CSF/HSA adducts. The use of two antibodies for capturing and detecting GM-CSF/HSA adducts eliminated potential cross-reactivities from either free GM-CSF or free HSA (Table I). Prior incubation of wells with GM-CSF significantly inhibited assay with GM-CSF/HSA adducts, so the samples should be freed of GM-CSF. The specificity of the capture antibody was shown by the lack of reactivity when ELISA was performed in wells coated either with unrelated antibody or with rabbit IgG (Table I). The specificity of the capture

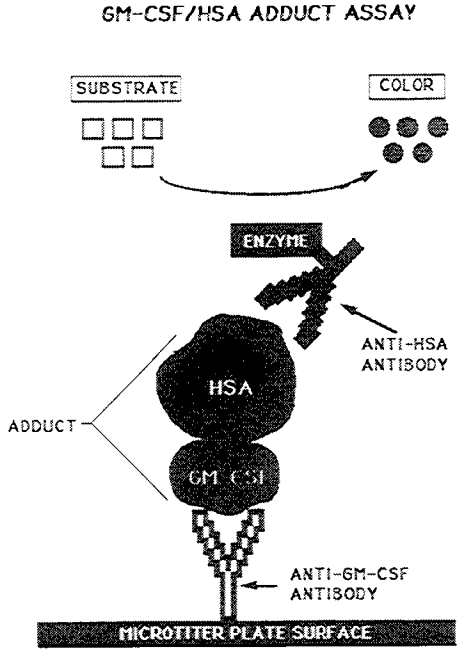

Fig. 5. Schematic of ELISA for GM-CSF/HSA adducts in solution mixtures. Most simplistic illustration of GM-CSF/HSA adduct formation at a 1:1 molar ratio.

Table I. Specificity of GM-CSF/HSA Adduct ELISA[a]

Sample	Concentration of protein (μg/mL)	Absorbance at 492 nm
HSA	100	0.040
GM-CSF	100	0.035
GM-CSF + HSA mixture[b]	50	0.035
GM-CSF + HSA mixture[c]	10	0.281
GM-CSF + HSA cross-linked with glutaraldehyde[d]	0.06	1.02
GM-CSF/HSA conjugates with SPDP[e]	0.3	1.4
Affinity-purified GM-CSF/HSA adducts[f]	0.03	1.10
Affinity-purified GM-CSF/HSA adducts (with nonimmune IgG capture antibody)	0.03	0.04

[a] ELISA was performed using different samples and comparing their relative absorbances. Unless indicated otherwise, the capture antibody was affinity-purified rabbit anti-GM-CSF and goat anti-HSA conjugated with peroxidase as the detector antibody.
[b] Solutions of GM-CSF (100 μg/mL) and HSA (100 μg/mL) were mixed and assayed.
[c] Solutions of GM-CSF (100 μg/mL) and HSA (100 μg/mL) were mixed, dialyzed against PBS overnight at RT, and assayed.
[d] GM-CSF and HSA were cross-linked with glutaraldehyde at a molar ratio of 1:1.
[e] GM-CSF/HSA conjugates were prepared as described under Materials and Methods.
[f] GM-CSF/HSA adducts purified by immunoaffinity chromatography as described under Materials and Methods.

antibody for GM-CSF-specific epitopes in GM-CSF/HSA adducts in the sample was also tested by competition binding experiments with free GM-CSF (data not shown).

Sensitivity and Linearity of the Assay

Absorbance values obtained for different concentrations of GM-CSF/HSA adducts in the reference standard yielded a sigmoidal curve (Fig. 6). During the evaluation of the reference standard, the detection limit was determined to be 0.25 ng of GM-CSF/HSA adducts. The workable range of quantitation of GM-CSF/HSA adducts in the assay is between 0.5 and 3 ng.

Recovery and Precision of the Assay

Several replicate solutions of HSA alone were spiked with a known amount of GM-CSF/HSA adduct and compared to GM-CSF/HSA adducts in buffer only. The recovery of spiked GM-CSF/HSA conjugated in samples ranged from 90 to 120%. The overall variability of ELISA for the reference standard and the sample measured as the relative standard deviation (RSD) ranged from 4.0 to 7.8% (Table II). However, the interday variation of the assaying samples of GM-CSF/HSA adducts formed by mixing solutions of GM-CSF and HSA (reproducibility) showed a much higher variability. The RSD ranged from 7.8 to 29%, when solution mixtures were analyzed on 3 different days (Table III). The variation could have been due to, among other things, sample preparation, which involved membrane filtration to remove monomeric GM-CSF in the sample.

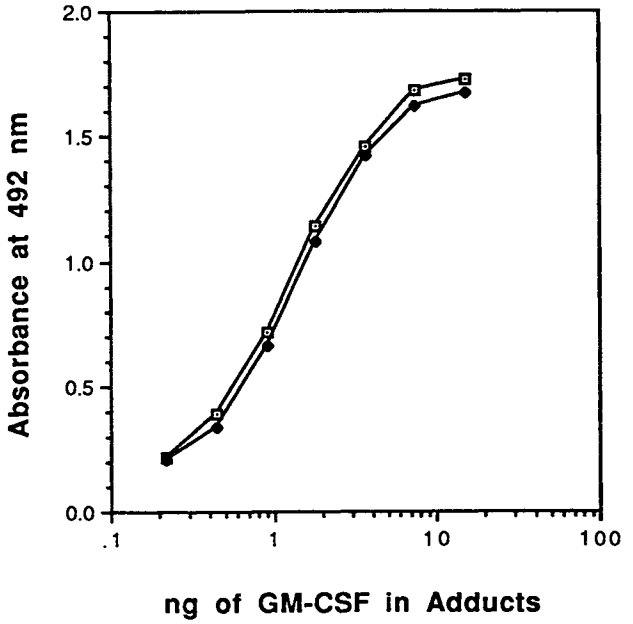

Fig. 6. ELISA standard curve for GM-CSF/HSA adducts in solution mixtures. (——□——) The reference standard isolated from GM-CSF and HSA solution mixture; (——■——) SPDP-mediated conjugates of GM-CSF/HSA.

SPDP-Mediated Cross-Linking of HSA with GM-CSF

Conjugates of GM-CSF/HSA were prepared to simulate the mercaptoethanol-reducible adducts found in solution mixtures of GM-CSF/HSA. Such conjugates could be used as a secondary reference standard in ELISA for screening samples. GM-CSF and HSA were ligated by disulfide bonds using SPDP, a heterobifunctional cross-linking reagent. First dithiopyridine groups were introduced into HSA by means of SPDP and reacted with reduced GM-CSF to give disulfide-mediated conjugates of GM-CSF/HSA. Based on the release of pyridine-2-thione as measured by its absorbance at 342 nm, approximately 2 or 3 molecules of GM-CSF were linked to each HSA molecule. The disulfide linkages of GM-CSF/HSA conjugates were sensitive to reducing conditions. The GM-CSF/HSA conjugates migrated as diffuse bands with a molecular weight >67,000 on SDS gels (Fig. 7). Fol-

Table II. Intraassay Precision of the ELISA

Sample no.[a]	μg of GM/HSA adducts			Mean	% RSD
1	1.40	1.40	1.60	1.46	7.8
2	1.60	1.60	1.80	1.66	6.9
3	2.50	2.40	2.60	2.50	4.0

[a] Samples were retentates obtained from membrane filtration of different solution mixtures of GM-CSF and HSA at the following ratios of GM-CSF to HSA: Sample 1, 1.0:1.0; Sample 2, 0.05:1.00; and Sample 3, 0.40:1.0. Three different dilutions of samples were assayed.

Table III. Interday Precision of the ELISA

Sample no.[a]	μg of GM/HSA adducts in solution mixtures				
	Day 1	Day 2	Day 3	Mean	% RSD
1	0.82	0.59	0.65	0.68	17.37
2	1.55	1.24	0.86	1.21	28.40
3	0.78	0.69	0.50	0.65	21.76
4	0.50	0.56	0.40	0.48	16.60
5	1.80	2.28	2.20	2.09	12.28
6	1.75	1.60	1.50	1.61	7.78
7	0.70	1.20	0.80	0.90	29.39

[a] Samples were retentates obtained from membrane filtration of different solution mixtures of GM-CSF/HSA at the following ratios of GM-CSF to HSA: Sample 1, 0.4:1.0; Sample 2, 0.05:1.0; Sample 3, 0.4:1.0; Sample 4, 1.0:1.0; Sample 5, 0.4:1; Sample 6, 0.4:1.0; Sample 7, 0.4:1.0.

lowing reduction with mercaptoethanol, GM-CSF was released from the conjugates (Fig. 7). In the ELISA system, the response curve generated by serial dilution of the chemically synthesized GM-CSF/HSA conjugates showed parallelism to the curve generated by adducts isolated from the GM-CSF/HSA solution mixtures (Fig. 6). Thus, the antibodies in the assay exhibit very similar affinities for the chemically synthesized adducts and the adducts isolated from solution mixtures of GM-CSF and HSA. The chemically synthesized adducts can also be used as an ELISA standard.

CONCLUSIONS

HSA is frequently used as a carrier and protective agent during storage and use of recombinant DNA-derived proteins. However, in this report it was found that, to a small extent, the HSA chemically combined with the recombinant DNA-derived molecule (GM-CSF). Because of concern about the possible effects of this complex on the patient, it is necessary to quantitate this chemical adduct. Quantification by SDS-PAGE or immunoblotting is precluded by the inaccuracies of staining and densitometry involved in these methods. The ELISA developed here utilized a unique system of anti-GM-CSF capture antibodies and anti-HSA detection antibodies. The ELISA was sensitive and specific for the adduct in the presence of free HSA.

The pathway for the production of GM-CSF/HSA adducts is still speculative. It is possible that conformational changes in HSA and GM-CSF induced by heat unfold the protein molecules. A reactive thiolate anion in HSA can attack disulfide bonds in GM-CSF, leading to the formation of reducible GM-CSF/HSA adducts. Examinations of the structures of GM-CSF and HSA show the availability of a free sulfhydryl group in HSA but not in GM-CSF. Of 35 cysteine residues in HSA, 34 are tied up in disulfide linkages, leaving 1 free cysteine (8,22). However, in GM-CSF, all four cysteine residues are linked by disulfide linkages (5). Thermal exposure has been shown to cause denaturation of the HSA molecule, with unfolding of the protein structure around the free sulfhydryl group of cysteine (23). Under alkaline or neutral conditions, the thiolate anion in HSA is active and might cause thiol disulfide interchange between HSA molecules and with the intact disulfide linkages of GM-CSF. The nature of the covalent bonding (other than the one arising from a reducible disulfide linkage) between GM-CSF and HSA is not known. It may arise from interaction between some reactive carbonyl and NH_2 groups in GM-CSF and HSA after prolonged contact.

The nature of the nonreducible chemical linkage between GM-CSF and HSA might be approached through the use of preparative electrophoresis and fast atom bombardment mass spectrometry. The stoichiometry of adduct formation also requires more investigation. While the linkage is simply understood as a 1:1 phenomenon, the electrophoresis experiments indicate that higher stoichiometries are present. The bioactivity of these adducts compared to the parent GM-CSF is under investigation.

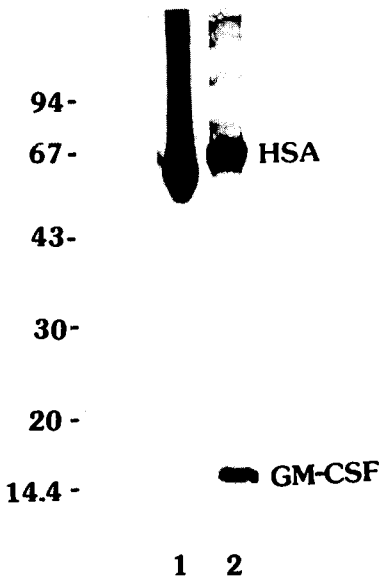

Fig. 7. SDS-PAGE analysis of SPDP-mediated conjugates of GM-CSF and HSA. Lane 1, 5 μg of protein conjugates run under nonreducing conditions; Lane 2, 5 μg of protein conjugates run under reducing conditions (i.e., with 2-mercaptoethanol).

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