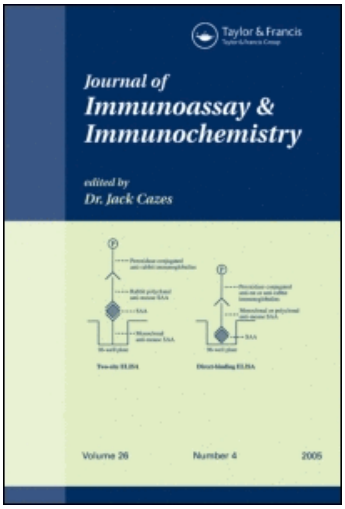

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A Cocktail of Three Monoclonal Antibodies Significantly Increases the Sensitivity of an Enzyme Immunoassay for Human Granulocyte-Macrophage Colony-Stimulating Factor

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A COCKTAIL OF THREE MONOCLONAL ANTIBODIES SIGNIFICANTLY INCREASES THE SENSITIVITY OF AN ENZYME IMMUNOASSAY FOR HUMAN GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR

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ABSTRACT

A sensitive and specific two-site ELISA was developed for human granulocyte-macrophage colony-stimulating factor (huGM-CSF) based on monoclonal antibodies (mAbs) which have been selected for high affinities and different epitope specificities. Using a cocktail of three mAbs, both for coating and, in their labeled form, for detection, a major increase in sensitivity was achieved (20-fold) compared to a two-site assay employing two different mAbs (one for coating and one for detection). The assay is as sensitive as the most sensitive biological assays. Recombinant mammalian cell expressed and natural huGM-CSF can be reliably detected down to 100 pg/ml (7 pmol/l). In contrast to conventional bioassays, the ELISA is highly specific for huGM-CSF and does not detect other human lymphokines. Results from quantification of recombinant and natural huGM-CSF in ELISA and bioassay correlate. (KEY WORDS: Monoclonal antibodies, Enzyme immunoassay, Granulocyte-macrophage colony-stimulating factor)

INTRODUCTION

Human granulocyte-macrophage colony-stimulating factor (huGM-CSF) is one of several glycoproteins that regulate the prolifera-

tion, differentiation and functional activation of hematopoietic cells (1). The availability of large quantities of recombinant material has permitted application of huGM-CSF as a therapeutic agent (2-5). Biotechnological production and clinical use of huGM-CSF require a rapid, sensitive and specific assay for quantification of huGM-CSF.

Therefore we developed a two-site or sandwich enzyme immunoassay for huGM-CSF based on epitope-mapped mAbs. Traditionally, two different mAbs are used in a sandwich assay, one mAb as capture antibody and the other mAb in a labeled form for detection (6). We show that a cocktail of three different mAbs used for both capture and detection significantly increased the sensitivity of the assay compared to combinations using only two mAbs.

MATERIALS AND METHODS

Cell Lines, Media and Supernatants

Chinese hamster ovary (CHO) cells transfected with the human GM-CSF gene and M-07 cells (7) were kindly provided by Genetics Institute, Cambridge, MA. CHO cells were cultured in Iscove's modified DMEM (Dulbecco's modified Eagle medium; Gibco, Paisley, Scottland) supplemented with 1% fetal calf serum (FCS; Boehringer, Mannheim, FRG), insulin (Collaborative Research, Lexington, MA), human transferrin (Behringwerke, Marburg, FRG), lecithin (Centrolex R lecithin, Central Soya Overseas BV, Rotterdam, The Nether-

lands), human serum albumin (Swiss Red Cross, Bern, Switzerland) and 0.1 μM methotrexate (Serva, Heidelberg, FRG). The human leukemia T cell line Jurkat was provided by Kendall Smith, Dartmouth Medical School, Hanover, NH. Jurkat cells ($2*10^6$/ml) were stimulated for 48 h with 1 μg/ml phytohemagglutinin and 20 ng/ml phorbol myristate acetate in Iscove's modified DMEM (8). The human bladder carcinoma cell line 5637 and the cutaneous T cell lymphoma HuT 78 were obtained from the American Type Culture Collection (Rockville, MD) and were grown in RPMI1640/10% FCS (Gibco) and Iscove's modified DMEM/5% FCS respectively.

Lymphokines

Purified recombinant huGM-CSF expressed by CHO cells (CHO-GM-CSF) and huGM-CSF COS supernatant was kindly provided by Genetics Institute, Cambridge, MA. Purified recombinant human IL-2, IL-3, IL-4, IL-6 and IL-5 COS supernatant were produced within the Biotechnology Research Area, Sandoz Ltd., Basle, Switzerland. Human IL-1α, human TNFα, murine IL-3, murine IL-4, murine GM-CSF, murine TNFα were purchased from Genzyme Corporation (Boston, MA), human Erythropoietin was from Amersham International (Little Chalfort, England).

Bioassay for huGM-CSF

M-07 cells were maintained in DMEM/10% FCS containing 10 U/ml human IL-3. Proliferation assays were performed with $2*10^4$ cells/well incubated for 72 h with huGM-CSF (30-0.01 U/ml) as standard

or appropriate dilutions of the samples. Proliferation was measured using the colorimetric assay described by Mosmann (9). Jurkat supernatant containing GM-CSF as well as IL-3 was tested in the presence of a neutralizing anti-IL-3 mAb (Lokker et al., submitted for publication).

Generation of anti-huGM-CSF mAbs

Eight-week-old female BALB/c mice (Madörin, Füllingsdorf, Switzerland) were immunized with 60 µg of purified CHO-GM-CSF, given in a 1:1 mixture of complete Freund's adjuvant (Difco Laboratories, Detroit, MI) and phosphate-buffered saline (PBS) in the hind footpad and subcutaneously in the neck. Four weeks later, the mice received the same amount of antigen in incomplete Freund's adjuvant (Difco). Two weeks after the second immunization 15 µg CHO-GM-CSF in incomplete Freund's adjuvant were injected intramuscularly and subcutaneously. Eleven days later, the mice were bled from the retroorbital venous plexus and the titer of the immune sera were determined in a solid-phase ELISA. Three mice selected for a high serum titer were boosted four weeks later with 50 µg huGM-CSF in PBS intravenously and intraperitoneally on days 4, 3, 2 and 1 prior to fusion (10) of the spleen cells with PAI-0, a derivative of the murine myeloma P3-X63-Ag8 (11). Fusions were performed essentially as described (12). Supernatants of growth-positive cultures were screened for specific antibodies in a solid-phase ELISA (see below). Specific hybridomas were cloned by limiting dilution and adapted to serum-free medium.

Purification and Biotinylation of mAbs

MAbs were purified from serum-free culture supernatants by affinity chromatography on Protein A (13). Purified antibodies (1 mg/ml) were dialyzed against 0.1 M $NaCO_3$ and reacted for 1 h at room temperature at a ratio of 1:5 (w/w) with a biotin succinimide ester with an extended spacer arm (Sulfosuccinimidyl 6-(biotin-amido) Hexanoate; Pierce Chemicals). The biotinylated mAbs were dialyzed and stored in PBS containing 0.02% NaN_3.

Enzyme Immunoassays

General procedure: Except where stated otherwise all reagents used in ELISA were diluted in PBS containing 1% bovine serum albumin (BSA; Fraction V; Boehringer). Reaction volumes were 100 µl per well, except for the blocking step which required 200 µl per well. Incubation times were 2-3 h at 37°C or overnight at 4°C, only streptavidin alkaline phosphatase was incubated for 1/2 h at room temperature. Washings between all incubation steps were done four times with PBS containing 0.05% Tween 20 (Merck-Schuchardt, Hohenbrunn, FRG).

Specificity of anti-huGM-CSF mAbs: CHO-expressed huGM-CSF (500 ng/ml) diluted in PBS was coupled to 96-well microtiter plates (Nunc-Immuno Plate II F, Gibco BRL AG, Basle, Switzerland). After blocking residual protein-binding sites with 2% BSA in PBS the plates were incubated with antisera ($1:10^3$ - $1:10^6$) and hybridoma supernatants (1:5 - 1:10). Bound antibodies were detected directly with a goat anti-mouse IgG alkaline phosphatase conjugate (1:1000; Sigma, St. Louis, MO) or indirectly with a rabbit anti-

mouse Ig antibody preparation (1 µg/ml; Nordic Immunological Laboratories, Tilburg, The Netherlands) plus a goat anti-rabbit alkaline phosphatase conjugate (1:1000; Sigma). One hour after addition of the substrate p-nitrophenyl phosphate (Sigma; 1 mg/ml in 0.1 M diethanolamine, pH 9.8) the optical density was measured at 405/492 nm. The absorbance difference between two wavelengths was chosen to eliminate the effects of non-specific absorbing substances.

Quantification of mAb concentrations in hybridoma supernatants: Serial dilutions of hybridoma supernatants (1:100 to 1:6400) and purified myeloma proteins (MOPC 21, IgG1/κ); UPC 10, IgG2a/κ; MOPC 141, IgG2b/κ and FLOPC 21, IgG3/κ; all from Sigma), or mAb (0-100 ng/ml) were incubated with solid-phase coupled goat anti-mouse IgG (4µg/ml; Sigma). Bound immunoglobulin was directly detected with a goat anti-mouse IgG alkaline phosphatase conjugate. The immunoglobulin content of the hybridoma supernatants was calculated by using purified myeloma proteins or mAb of the same isotype as standards.

Affinity determination: The affinities of anti-huGM-CSF mAb were determined by incubation of four different concentrations of solid-phase coupled CHO-GM-CSF (50, 25, 12.5 and 6.25 ng/ml) with serial dilutions (0.015-16000 ng/ml) of antibody preparations or hybridoma supernatants with known immunoglobulin content. Antibody affinity constants (K) were calculated as described by Beatty et al. (14) using the formula $K = (n-1)/2(n[Ab']_t - [Ab]_t)$ where $n=[Ag]_t/[Ag']_t$ and $[Ab]_t$ and $[Ab']_t$ are the concentration of total

added antibody giving 50% of maximal binding at two different antigen concentrations ($[Ag]_t$ and $[Ag']_t$).

Mutual inhibition: Solid-phase coupled CHO-GM-CSF (6.25 ng/ml) was incubated with serial dilutions of unlabeled mAbs. After 1 h a constant amount of biotinylated mAbs (125 ng/ml) was added resulting in a ratio of unlabeled to labeled mAb of 0.1 - 30. Bound biotinylated mAbs were detected with a streptavidin alkaline phosphatase conjugate (1:7000; Jackson ImmunoResearch Laboratories, Avondale, PA). The ratio of unlabeled to labeled mAbs required for 50% inhibition was calculated.

Sandwich ELISA: Either one mAb (5 µg/ml) or a cocktail of three mAbs (1.7 µg/ml of each mAb) were coated to solid-phase and subsequently incubated with dilutions of purified CHO-GM-CSF standard or samples. Bound huGM-CSF was detected by incubation with either one biotinylated mAb (200 ng/ml) or a cocktail of three mAbs (200 ng/ml of each mAb) and streptavidin alkaline phosphatase (1:7000). The protein content of the CHO-GM-CSF standard had been determined by quantitative amino acid analysis (15). For specificity control the following lymphokines were used: huIL-1α (100 U/ml), huIL-2 (1000 ng/ml), huIL-3 (1000 ng/ml), huIL-4 (1000 ng/ml), huIL-5 (COS supernatant), huIL-6 (1000 ng/ml), huG-CSF (300 U/ml), hu erythropoietin (100 U/ml), huTNFα (100 U/ml), mIL-3 (500 U/ml), mIL-4 (100 U/ml), mGM-CSF (200 U/ml) and mTNFα (100 U/ml). The sandwich ELISA using the cocktail of three mAbs was validated by adding 10, 30 and 90 ng/ml to RPMI medium containing 10% FCS. Serial dilutions of each sample were assayed on the three plates per day on three different days.

RESULTS

Generation of anti-huGM-CSF mAbs and Selection of mAbs for the Immunoassay

Fusions were screened for mAbs with specificity for huGM-CSF in solid-phase ELISA. In order to select mAb with high affinity for huGM-CSF the immunoglobulin content of supernatants from 39 cloned hybridomas was determined in solid-phase ELISA using purified, isotype-matched myeloma proteins or mAb as standards. All supernatants were adjusted to the same immunoglobulin content and titrated on a constant amount of huGM-CSF. The amount of mAb required for 50% of maximal binding varied between >5000 ng/ml and 1 ng/ml (data not shown). Six mAbs which show the highest relative affinity in this assay (low mAb concentration required for half maximal binding) were selected. They are of the IgG1/κ isotype. The affinity constants, determined in solid-phase ELISA according to Beatty et al. (14) vary between 0.6 and $6.4*10^9$ l/mol (Table 1).

Epitope Mapping by mutual Inhibition

For the establishment of a sandwich ELISA, mAbs recognizing different epitopes should be used for coating and detection. The relative positions of the epitopes of the selected mAbs were mapped on the surface of the huGM-CSF molecule by competitive binding of mAbs to solid-phase coupled huGM-CSF. Three epitopes could be distinguished (Table 1). Only cases, where an equal inhibition of the mAbs was found in both directions are considered.

TABLE 1

Epitope Mapping of anti-GM-CSF monoclonal Antibodies by mutual Inhibition in solid-phase ELISA

MAb-biotin	K[a] [l/mol]	Inhibitor							Epitope
		5	92	173	315	98	59	746	
5	$1.7*10^9$	0.4[b]	>30	>30	>30	>30	>30	>30	A
92	$0.6*10^9$	>30	1.5	1.1	30.0	1.7	>30	>30	B
173	$0.9*10^9$	>30	1.7	1.3	16.0	1.8	>30	>30	B
315	$5.1*10^9$	>30	>30	>30	1.9	6.0	7.0	>30	C
59	$5.7*10^9$	>30	>30	>30	5.5	2.5	0.4	>30	C
98	$6.4*10^9$	>30	>30	>30	1.4	0.8	0.3	>30	C

[a]Antibody affinity constants (K) were calculated according to Beatty et al. (14).
[b]Ratio of unlabeled to labeled mAb required for 50% inhibition.

MAb 5 is not inhibited by a 30-fold excess of the other mAbs and thus defines an epitope (A) which seemed to be separate from all the others. The epitopes of the mAbs 92 and 173 are identical or at least very closely related because the corresponding mAbs mutually inhibit (epitope B). A third distinct epitope (C) is defined by mAbs 59, 98 and 315 which compete with each other but not with the remaining three mAbs. No inhibition was observed with a 30-fold excess of an isotype-matched control mAb (746) of unrelated specificity.

TABLE 2

Establishment of the Sandwich-ELISA
using different Combinations of monoclonal Antibodies[a]

Coating	Detection					
mAb	5	92	173	315	59	98
5	0.010[b]	0.245	0.269	0.161	0.089	0.055
92	0.029	0.015	0.010	0.719	0.946	0.030
173	0.019	0.016	0.011	0.487	1.052	0.026
315	0.110	1.063	1.093	0.025	0.015	0.012
59	0.020	0.778	0.909	0.013	0.012	0.010
98	0.018	0.031	0.025	0.011	0.012	0.008

[a]MAbs were coated to solid-phase and incubated with 100 ng/ml hu-GM-CSF. Bound GM-CSF was detected with biotinylated mAbs.
[b]Absorbance 405/492 nm after 1 h of incubation with substrate. Means of duplicate determinations are shown.

Establishment of a Sandwich ELISA

We tried all possible combinations of mAbs by coating with an unlabeled mAb, then incubating with a fixed amount of huGM-CSF (100 ng/ml) and detecting with an labeled mAb (Table 2). Combinations where the mAb of the same epitope cluster were used for coating as well as for detection could not detect any huGM-CSF presumably due to steric hindrance. Combinations where mAb 5 as the only epitope cluster A-specific mAb is included showed a weak signal with 100 ng/ml huGM-CSF. The best signals were obtained with mAbs recognizing epitope B in combination with mAbs recogni-

zing epitope C. The absorbance values ranged from 0.5 (using the combination of mAbs 173/315-biotin) to 1.1 (315/173-biotin). MAb 98 used either for coating or for detection could not detect huGM-CSF in this sandwich type of ELISA. The epitope of this mAb seemed to be present only on huGM-CSF coupled to ELISA-plates, indicating a partial change of the conformation of the huGM-CSF molecule when absorbed to plastic surfaces.

Increasing the Sensitivity of the Sandwich ELISA

We tried to increase the sensitivity of the sandwich ELISA by using cocktails of mAbs for coating and for detection. Titrating CHO-GM-CSF in the most sensitive combination of two mAbs with different epitope specificities (315/173-biotin, see Table 2) resulted in a half maximal binding at 38 ng/ml huGM-CSF (Fig. 1). A cocktail of three mAbs (5, 173 and 315) recognizing three different epitopes (Table 1) used for coating and in their labeled form for detection increased the sensitivity 20-fold (half maximal binding at 1.9 ng/ml; Fig. 1). Comparison of cocktails containing all possible combinations of mAbs with specificities for the three different epitopes showed no difference in sensitivity (data not shown). The sensitivity of this assay averaged 20 - 30 pg/ml (1.4 - 2.1 pmol/l; Fig. 1). Any signal greater than two times that of assay buffer was considered as positive.

Specificity of the huGM-CSF Sandwich ELISA

The specificity of the sandwich ELISA for huGM-CSF is shown by the lack of reactivity with 13 lymphokines of human and murine

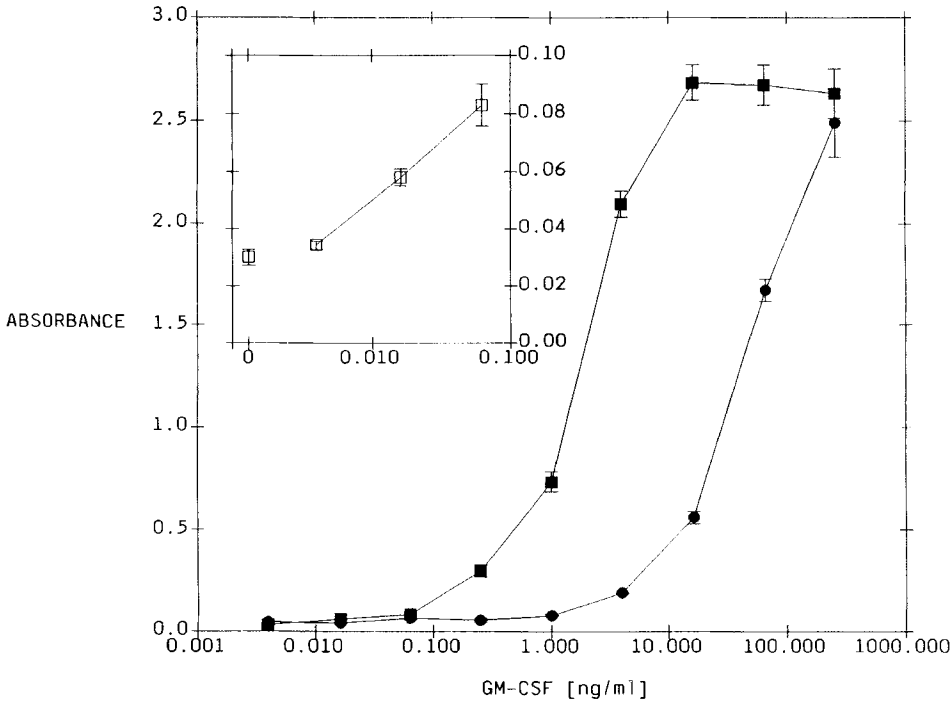

FIGURE 1. Sensitivity and specificity of the GM-CSF sandwich ELISA. Either one mAb (315: ●) or three mAbs (5, 173 and 315: ■) were coated to ELISA plates and incubated with huGM-CSF. Bound GM-CSF was detected either with biotin labeled mAb 173 (●) or mAbs 5, 173 and 315 (■). The inset shows an amplification of the lower part of the curve obtained with the cocktail. Means and standard errors of triplicate determinations are shown.

origin (Table 3). There is no cross-reactivity with mGM-CSF (200 U/ml), the only lymphokine reported to show homology with huGM-CSF (16). Using the sandwich ELISA significant amounts of GM-CSF could be detected in supernatants of three different human cell lines (Jurkat, HuT 78 and 5637; Table 3). In all cases the sample dilutions parallel the standard curve.

TABLE 3

Specificity of the GM-CSF Sandwich ELISA

Sample[a]	GM-CSF [pg/ml]
huGM-CSF CHO supernatant	5,250,000
huGM-CSF COS supernatant	2,375,000
human IL-1α (100 U/ml)	<30
human IL-2 (1000 ng/ml)	<30
human IL-3 (1000 ng/ml)	<30
human IL-4 (1000 ng/ml)	<30
human IL-5 (COS supernatant)	<30
human IL-6 (1000 ng/ml)	<30
human G-CSF (300 U/ml)	<30
human Erythropoietin (100 U/ml)	<30
human TNFα (100 U/ml)	<30
murine IL-3 (500 U/ml)	<30
murine IL-4 (100 U/ml)	<30
murine GM-CSF (200 U/ml)	<30
murine TNFα (100 U/ml)	<30
Jurkat supernatant	4,460
HuT 78 supernatant	770
5637 supernatant	720
Medium	<30

[a]Samples were assayed in a sandwich ELISA for GM-CSF utilizing a cocktail of three mAbs (5, 173 and 315) for coating and detection as described in Materials and Methods.

TABLE 4

Accuracy and Precision of the Sandwich ELISA[a]

Sample [ng/ml]	Accuracy		Intra-assay Precision	Inter-assay Precision			
	GM-CSF detected [ng/ml]	Recovery [%]	Mean CV[b] [%]	Plate to Plate Variation CV [%] Day 1	Day 2	Day 3	Day to Day Variation CV [%]
10	10.3	103	2.7	6.5	0.5	2.7	2.6
30	30.8	103	3.5	7.7	2.5	2.4	6.8
90	92.4	103	3.2	5.9	1.7	2.4	3.8

[a]Serial dilutions of three GM-CSF samples in RPMI/10% FCS were assayed on three plates per day on three days.
[b]Coefficient of variation.

Accuracy and Precision of the Sandwich ELISA

The sandwich ELISA quantitatively measures huGM-CSF in an accurate and precise manner (Table 4). Recovery was close to 100%. Both, intra- and inter-assay variations were below 10%.

Correlation between Sandwich ELISA and M-07-Bioassay

Several samples containing recombinant (CHO- and COS-expressed) as well as natural (5637 and Jurkat supernatant) huGM-CSF were tested in parallel in the sandwich ELISA and in the M-07 bioassay. A good correlation (r=0.985) between the immunoassay and the bioassay was found for all samples (Fig. 2).

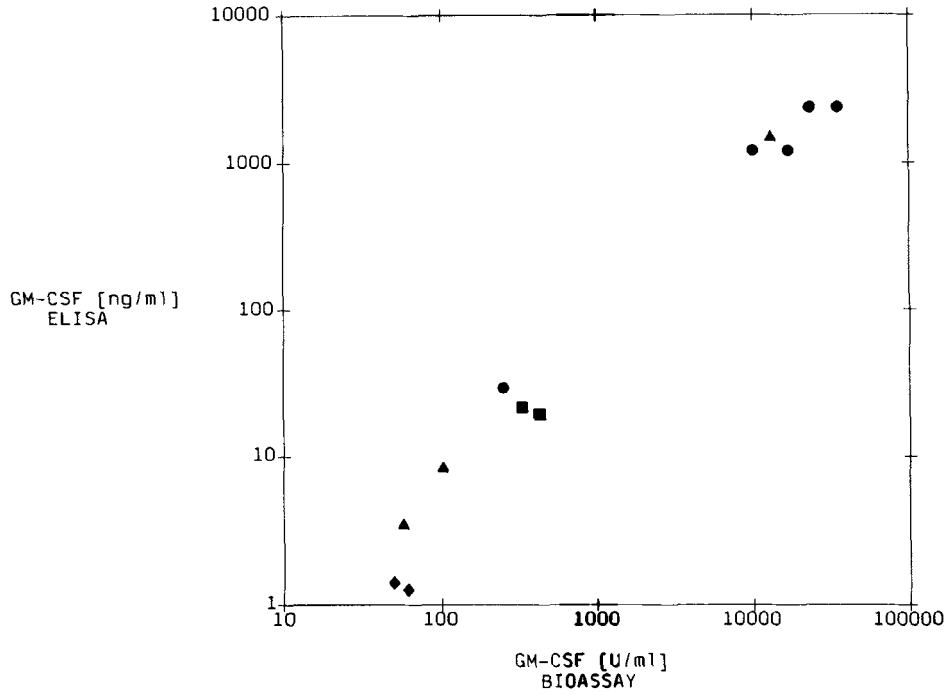

FIGURE 2. Correlation of huGM-CSF concentrations measured by bioassay (M-07) and immunoassay (sandwich ELISA). Supernatants containing recombinant huGM-CSF (CHO-expressed, ▲; COS-expressed, ●) or natural huGM-CSF (5637, ◆ ; Jurkat, ■) were assayed by both methods. The results were correlated by linear regression analysis (r=0.985).

DISCUSSION

At first huGM-CSF quantification was achieved by biological assays using colony formation in semisolid medium of either bone marrow cells or a myeloid leukemia cell line (16). Later more rapid proliferation assays were developed using primary leukemic cells isolated from patients with chronic myelogenous leukemia or

cell lines established from patients with acute myeloblastic leukemia or T-cell leukemia (17-19). All biological assays are highly sensitive for huGM-CSF (picomolar range) but are not absolutely specific for huGM-CSF; they respond to other lymphokines as well (20-22).

An alternative assay system for huGM-CSF would be a competitive receptor binding assay. Although a single class of high affinity receptor specific for huGM-CSF exist on mature primary cells as well as on undifferentiated leukemia cell lines, this type of assay is hampered by the low number of receptors (<1000) present on GM-CSF responsive cells (23,24). Thus, immunoassays using huGM-CSF specific antibodies are the method of choice.

We developed an immunoassay solely based on mAbs to avoid the disadvantages of polyclonal antisera with regard to reproducibility in titer, affinity and specificity. Sandwich immunoassays appear to be more sensitive than competitive assays (25). The sensitivity of a sandwich ELISA is mainly determined by the affinity of the antibodies used (26). Thus the immunization schedule and primary selection of mAbs were designed to obtain mAbs of the highest possible affinity. The second selection step was based on epitope specificity in order to select mAbs which do not mutually inhibit their binding to the antigen. Identification of mAbs recognizing different epitopes offered the possibility of establishing a sandwich ELISA using combinations of two different mAbs (Table 2). The sensitivity of the sandwich ELISA was substantially increased (20-fold) by using cocktails of three mAbs with differ-

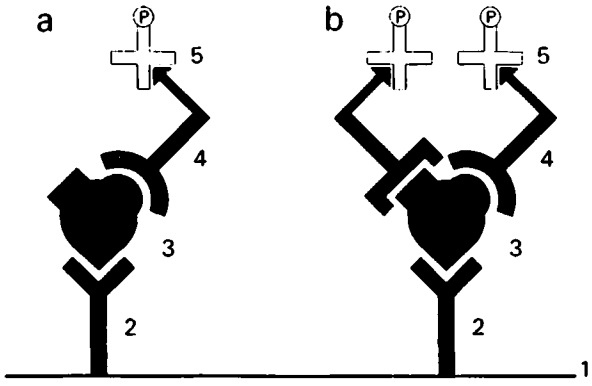

FIGURE 3. Diagram to illustrate how the use of a mAb cocktail can increase the sensitivity of a sandwich ELISA. (a) Sandwich ELISA with two different mAb. (b) Sandwich ELISA using three different mAbs. (1) Solid phase. (2) Epitope-mapped mAb used for coating. (3) huGM-CSF with three different epitopes. (4) Biotinylated, epitope-mapped mAb used for detection. (5) Streptavidin-alkaline phosphatase conjugate.

ent epitope specificities for coating and detection (Fig. 1). This can be explained by the simultaneous binding of two different mAbs to one huGM-CSF molecule captured by one mAb (illustrated in Fig. 3).

The detection limit for huGM-CSF in our assay was on average 20-30 pg/ml (1.4-2.1 pmol/l), allowing reliable quantification of huGM-CSF down to 100 pg/ml (7 pmol/l) which is in the range of the most sensitive bioassays (21).

To date four immunoassays, two RIA and two ELISA have been described for the detection of huGM-CSF. Using rabbit antisera against yeast or COS cell expressed recombinant huGM-CSF the RIA reached a sensitivity in the low nanogram range (27,28). Both

ELISA use one solid-phase coupled mAb to capture the antigen and polyclonal rabbit antibodies for detection. One ELISA could detect 10 - 90 ng/ml of yeast expressed recombinant huGM-CSF in BSA/PBS solution (29) whereas the second ELISA is sensitive to 20 pg/ml huGM-CSF in human serum (30). As the most sensitive ELISA for huGM-CSF reported so far uses a single mAb for capture the subsequent detection is solely dependent on the presence of a single epitope, which might be destroyed by metabolic processes or affected by serum components. A sandwich ELISA consisting of several mAbs should be less influenced by metabolic modifications of GM-CSF or by components present in the samples to be assayed. Thus, our sandwich ELISA might be suitable for detection and quantification of GM-CSF in biological fluids as well as in human blood samples in healthy and diseased states.

The use of a mixture of mAbs may lead to a systematic bias when the assay involves the quantification of multiple structurally similar antigens like isoforms of enzymes and subclasses of immunoglobulins. This seems to be rather unlikely for huGM-CSF since there is no cross-reactivity with mouse GM-CSF which is the only lymphokine with some homology to huGM-CSF.

We have developed a sensitive and specific sandwich ELISA for the detection of huGM-CSF which is entirely based on mAbs. Using a cocktail of three epitope-mapped mAbs we could significantly increase the sensitivity of the assay compared to an ELISA consisting of two different mAbs. This approach may be generally applicable to enhance the sensitivity of two-site ELISA systems.

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