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Mapping of the antibody- and receptor-binding domains of granulocyte colony-stimulating factor using an optical biosensor

Comparison with enzyme-linked immunosorbent assay competition studies

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ABSTRACT

An automated optical biosensor instrument for measuring molecular interactions (Pharmacia BIAcore) has been used to characterise the epitopes recognised by 15 monoclonal antibodies raised against recombinant human granulocyte colony-stimulating factor (G-CSF). The BIAcore combines an autosampler and integrated microfluidic cartridge for the introduction and transportation of samples to the sensor chip surface, with surface plasmon resonance to detect binding events. A rabbit anti-mouse Fc antibody, coupled to the sensor surface *in situ* using conventional protein chemistry techniques, was used to capture an anti-G-CSF monoclonal antibody. G-CSF was bound to this antibody by injection over the sensor surface. Multi-site binding experiments were then performed in which other anti-G-CSF monoclonal antibodies were injected sequentially over the surface, and their ability to bind to the G-CSF in a multimolecular complex monitored in real time. Results obtained using the

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biosensor have been compared with data obtained by cross competition studies using biotinylated antibodies or antibody binding studies using chemically or enzymatically derived G-CSF peptide fragments or synthetic peptides. The results of these studies are in excellent agreement with the data from the BIAcore, although modification of the antibody or G-CSF occasionally altered the epitope affinity.

INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is one of a family of hemopoietic growth factors which regulate proliferation and differentiation of hemopoietic precursor cells, as well as the activity of mature neutrophils [1]. G-CSF has been purified from several species [2,3]. N-terminal and internal amino acid sequence data has allowed the isolation of the corresponding cDNA [4,5] and subsequent production of recombinant human G-CSF (hG-CSF) for structural studies [6] and therapeutic use [7].

The tertiary structure of G-CSF has been elucidated from X-ray crystallography studies [8], which have shown G-CSF, as predicted by Parry *et al.* [9] and Bazan [10], to belong to the four α-helical bundle group of growth factors typified by growth hormone [11]. However, the precise location of the residues involved in binding of G-CSF to the receptor are not known. In order to define functionally important regions of the molecule we have raised more than thirty monoclonal antibodies to recombinant hG-CSF and identified the amino acid sequence of each epitope [12]. Antibodies recognising similar epitopes were identified by enzyme-linked immunosorbent assay (ELISA) competition assays and characterised using neutralisation assays, conformational dependence and cross reactivity with canine G-CSF (cG-CSF) [12]. Herein we report biosensor studies on fifteen of these antibodies which demonstrate the speed and reliability of BIAcore analyses. The BIAcore is an automated instrumental biosensor [13] which employs surface plasmon resonance detection and enables rapid quantitation and characterisation of the interaction between monoclonal antibodies and their epitopes.

A biosensor can be defined as a device that combines a biological recognition mechanism with a suitable transducer, which generates a measurable signal in response to changes in concentration of a given biomolecule at the detector surface [14]. The biosensor used in these studies (Pharmacia BIAcore) combines sensor chip technology with the detection principle of surface plasmon resonance [13,15–18]. Changes in surface concentration directly correlate with changes in refractive index, which in turn are related to the angle at which surface plasmon resonance occurs, allowing for sensitive real time measurements of protein-protein interactions. The sensor chip consists of a glass slide coated uniformly with a thin (50 nm) gold surface. A carboxymethylated dextran polymer is attached to the surface [19] which increases the surface capacity, provides a hydrophilic environment suitable for studies of biomolecular interactions and allows for covalent linkage of target biomolecules using conventional protein chemistry reagents (*e.g.* N-hydroxysuccinimide–N-ethyl-N′-(3-diethylaminopropyl) carbodiimide [20]). This coupling can be performed *in situ* using the autosampler and integrated microfluidics of the instrument. Monoclonal antibodies, purified receptors, protein or peptide ligands may be readily coupled to the surface, and real time binding studies performed by flowing reagents of interest over the sensor surface to which the target proteins have been attached.

We demonstrate the use of this technology to compare the epitope specificities of 15 monoclonal antibodies raised against recombinant hG-CSF using multideterminant binding analyses, and compare the results obtained using the biosensor with ELISA competition studies using the same reagents [12].

MATERIALS AND METHODS

Instrumentation and reagents

All measurements were performed on the BIAcore biosensor (Pharmacia Biosensor, Uppsala, Sweden). Sensor chip CM5, Surfactant P20 (a 10% solution of a non-ionic detergent), N-

hydroxysuccinimide (NHS), N-ethyl-N′-(3-diethylaminopropyl) carbodiimide (EDC), ethanolamine hydrochloride, a rabbit anti-mouse Fc antibody (RAM Fc), subclass specific antibodies rabbit anti-IgG1, anti-IgG2a, anti-IgG2b and anti-IgG3 and an anti-α foetal protein (anti-AFP) monoclonal antibody were also from Pharmacia Biosensor. Recombinant hG-CSF was a kind gift from Dr. L. Souza (AMGEN, Thousand Oaks, CA, USA).

Monoclonal antibodies (MAbs) against recombinant hG-CSF were produced as described previously [12]. Antibodies were purified from either tissue culture supernatant or ascitic fluid by chromatography on Protein A-Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden). The antibody isotype was determined using the BIAcore [13]. All antibodies were found to be IgG1, except MAb 349 which was an IgG2b antibody.

Immobilisation of rabbit anti-mouse Fc antibody to the sensor surface

The RAM Fc antibody was immobilised onto the sensor surface essentially as described previously [13,19,20]. Briefly, immobilisation was performed at a constant flow-rate of 5 μl/min using HBS [10 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), pH 7.4, 0.15 *M* NaCl, 3.4 m*M* ethylenediaminetetraacetic acid (EDTA), 0.05% Surfactant P20. The carboxymethylated surface of the sensor chip was first activated with 25 μl of a NHS–EDC mixture (0.05 *M* NHS, 0.2 *M* EDC in distilled water). A 35 μl aliquot of RAM Fc antibody (100 μg/ml in 10 m*M* sodium acetate buffer, pH 4.5) was then injected over the activated surface. Following the coupling of RAM Fc to the sensor surface, residual unreacted active esters were blocked by the injection of 35 μl of ethanolamine–HCl, pH 8.5, followed by the removal of noncovalently bound antibody by injection of 15 μl of 10 m*M* HCl (Fig. 1).

Multideterminant binding analysis

Using the RAM Fc antibody which had been immobilised to the sensor chip as a general "entrapping" surface, multideterminant binding analyses of the anti-hG-CSF antibodies were performed at a constant flow-rate of 5 μl/min using the HBS buffer. A 25-μl aliquot of the primary monoclonal antibody (diluted to 100 μg/ml in HBS buffer) was injected over the RAM Fc surface. Following this pulse, 25 μl of an anti-AFP antibody (100 μg/ml in the HBS buffer without NaCl) was injected, to block any residual activated sites. The antigen, hG-CSF (15 μl, 50 μg/ml in HBS buffer), was then injected for binding to the entrapped primary antibody. Other antibodies (15 μl, 100 μg/ml in HBS buffer) were injected in sequence before regeneration of the sensor surface with 10 m*M* HCl (15 μl). Up to 4 antibodies were tested sequentially.

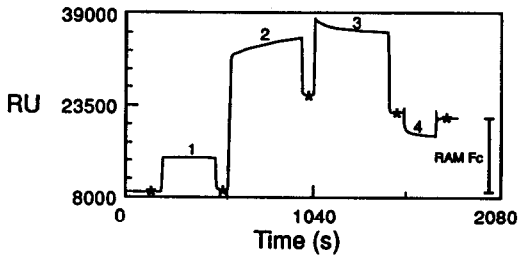
RESULTS AND DISCUSSION

The initial requirement for biosensor analysis is to immobilise the affinity reagent to the sensor surface. This reagent may be either one of the reactants in the interaction under investigation, or an antibody which can specifically capture one of the reactants without interfering with subsequent antigen interactions. In this study we have chosen to use a "general" entrapping antibody (RAM Fc), directed towards the conserved Fc portion of the murine monoclonal antibodies. The Fc region is not involved in ligand binding [21], and hence binding via this region does not interfere with the epitope specificity studies. The use of this antibody, rather than a subclass specific antibody (*e.g.* RAM G1) allows monoclonal antibodies of various subclasses to be compared under similar experimental conditions. The RAM Fc immobilisation was performed *in situ* automatically (Fig. 1A) and monitored using the surface plasmon resonance (SPR) detector. The SPR detector responds to changes in refractive index at, or close to, the sensor surface as manifested by changes in the angle at which surface plasmon resonance occurs. These changes are monitored continuously over time, and are recorded as a sensorgram (Fig. 1B). The differential in signal (resonance units, RU) between the initial baseline and the final steady state reading (12352 RU, Fig. 1C) was a function of the amount of covalently bound RAM Fc, and corresponded to a surface coupling density of approximately 12

(A) Immobilisation Program

FLOW	5				
TRANSFER	r2e1	r2e3	70	IEDC	
TRANSFER	r2e2	r2e3	70	INHS	
MIX	r2e3		115		
INJECT	r2e3		25	I1.EDC/NHS	(Activate surface)
INJECT	r2e4		35	I2.RAM Fc	(Couple)
INJECT	r2e5		35	I3.Ethanolamine	(Deactivate)
INJECT	r2f3		15	I4.10mM HCl	(Remove non-covalently bound material)

(B) Sensorgram



(C) Results

Time (s)	AbsResp	RelResp	Id
141.5	8959	0	baseline
1783.5	21311	12352	RAM Fc

Fig. 1. The immobilisation of RAM Fc antibody to the sensor surface. The RAM Fc antibody was covalently attached to the sensor surface using the program shown (A). The flow-rate of HBS was constant at 5 μl/min throughout the immobilisation procedure. A 70-μl volume of EDC and NHS was transferred from their respective positions in the autosampler rack (designated r2e1 and r2e2 respectively) and transferred to an empty vial (r2e3) for mixing. A 25-μl volume of this mixture was injected over the sensor surface to activate the carboxymethylated dextran surface of the chip. The signal observed during the activation is shown in the associated real time sensorgram (B). The initial positive reponse (signal 1) is due to the difference in refractive index between the EDC–NHS and HBS. Between injections, when HBS is flowing over the sensor surface, steady state readings are reported, and the relative response due to binding interactions quantitated. Following activation of the surface, 35 μl of RAM Fc antibody (100 μg/ml in 10 m*M* sodium acetate, pH 4.5) were injected (signal 2). At the end of this pulse a clear positive response (25100 RU) was observed. Following blocking of residual active sites with 35 μl of 1 *M* ethanolamine hydrochloride, pH 8.5 (signal 3) and removal of any residual non-covalently bound material with a pulse of 15 μl of 10 m*M* HCl (signal 4) the increase in signal due to the immobilisation of the RAM Fc was measured (C). The relative response of 12352RU corresponds to a surface concentration of approximately 12.4 ng/mm^2.

ng/mm^2 [22]. The total time required for the immobilisation procedure was less than 35 min.

Using the immobilised RAM Fc sensor surface, antibody cross-competition was investigated using multideterminant binding analyses. Control studies were performed (Table I) to investigate the concentration of antigen required for binding to the primary antibody, and also to enable the unambiguous detection level for positive binding to be determined. An anti-G-CSF monoclonal antibody (MAb 201) was trapped via the RAM Fc, and then residual RAM Fc sites were blocked using the anti-AFP antibody (an unrelated antibody of the same subclass) to prevent direct non-specific binding of the sequentially injected anti-G-CSF antibodies to the sensor surface. The concentration (100 μg/ml) and volume (35 μl) of the anti-AFP antibody

TABLE I

CONTROL BINDING STUDIES PRIOR TO MULTIDETERMIMINANT ANALYSES

All analyses were performed, using the RAM Fc surface, at a flow-rate of 5 μl/min and a temperature of 25°C. The injection volumes for the primary antibody and the anti-AFP blocking antibody were 25 μl. The injection volumes for the subsequent injections were 15 μl. The relative detector responses, read between injections when HBS is flowing over the sensor surface, are reported. The values in parentheses are the absolute responses for binding of G-CSF obtained by subtraction of the negative signal observed when buffer was injected instead of G-CSF (Experiment B).

	Step	Relative response
A	Baseline	0
	MAb 201	1529
	anti-AFP	1530
	G-CSF (50 μg/ml)	103 (232)
	MAb 204	443
	MAb 205	738
B	Baseline	0
	MAb 201	1568
	anti-AFP	1572
	Buffer	−129 (0)
	MAb 204	81
	MAb 205	141
C	Baseline	0
	MAb 201	1583
	anti-AFP	1452
	G-CSF (10 μg/ml)	−79 (50)
	MAb 204	198
	MAb 205	404

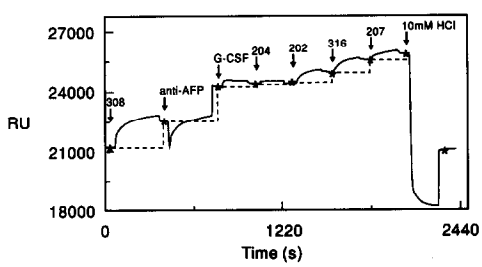
were chosen so that this unrelated antibody would be capable of saturating the RAM Fc surface in the absence of primary antibody. The total SPR signal observed after a saturating injection of the blocking antibody was 3041 RU (data not shown). This was similar to the combined SPR signal observed for both the anti-G-CSF monoclonal antibody and the blocking anti-AFP antibody used during the multisite-binding analyses (3059, 3140, 3035 RU in Table I, A, B, C respectively) and was independent of the identity of the primary antibody. This confirmed that the first MAb and the blocking antibody together occupy all the available binding sites.

When hG-CSF was injected at a concentration of 50 μg/ml, MAbs 204 and 205 clearly bound to the hG-CSF in the presence of the primary monoclonal antibody immobilised via the RAM Fc (responses 443 and 738RU respectively), indicating that all three antibodies recognise different epitopes (Table I, A). When buffer alone was injected following loading with the anti-G-CSF monoclonal antibody (MAb 201), the non-specific responses following injection of MAb 204 and MAb 205 were 81 and 141 RU, respectively (Table I, B). Typically non-specific responses were less than 150 RU, and this value was therefore chosen as the cut-off level to distinguish between negative and positive binding. However, when the concentration of hG-CSF was lowered to 10 μg/ml the response observed with MAb 204 (198 RU) approached the cut-off value of 150 RU (Table I, C). To facilitate interpretation of positive binding an antigen concentration of 50 μg/ml was used in all subsequent experiments.

It should also be noted that a negative relative response (−129 RU) was obtained following the injection of buffer (Fig. 1B). This was due to some slight dissociation of the anti-G-CSF and anti-AFP antibodies from the RAM Fc. The relative response observed for the G-CSF at 10 μg/ml (−79 RU, Fig. 1C) corresponded to an absolute signal of 50 RU above the buffer control, whilst the signal observed for hG-CSF at 50 μg/ml (103 RU, Fig. 1A) corresponded to an absolute signal of 232 RU. However, the use of an automated system rendered the dissociation constant between runs, so relative responses were used for all comparative evaluations.

It can be seen that the signal obtained upon binding of the ligand was considerably smaller than the signal obtained when the monoclonal antibody bound to the RAM Fc. This is because the detector is mass sensitive, and hence the relative signal that would be observed is a function of the relative molecular masses of the interacting species. Thus, in the example given in Table I, A (relative response of 1529 RU on binding of MAb 201 to the RAM Fc on the sensor surface), if one calculates a M_r of 19 000 for hG-CSF [6] and 150 000 for the MAb, and if one assumes that the antibody is bivalent [23,24], the maximum signal that could be expected on a 1:2 binding between the antibody and the ligand is 387 RU [(1529 · 19 · 2)/150]. The absolute value of 232 RU obtained for the binding of G-CSF to MAb 201 represents 60% maximum binding.

A typical multi-site binding analysis is depicted in Fig. 2, in which four anti-G-CSF monoclonal antibodies were injected sequentially following the hG-CSF. In this case the first of the sequentially tested antibodies (MAb 204) clearly failed to bind to hG-CSF which was attached via MAb

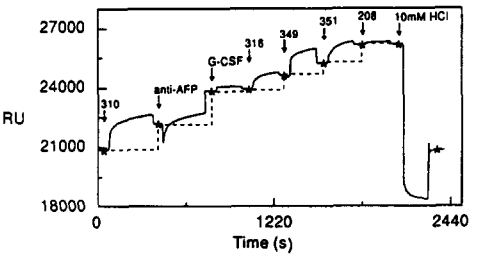


Time (s)	AbsResp	RelResp	Id	Time (s)	AbsResp	RelResp	Id
47.0	21233	0	baseline	1567.0	24952	568	202
413.0	22564	1331	308	1826.0	25576	624	316
792.0	24270	1706	anti-AFP	2086.0	25867	291	207
1050.0	24386	117	G-CSF	2346.0	21076	-4790	Reg.
1309.0	24384	-2	204				

Fig. 2. Sensorgram of a multideterminant binding analysis using MAb 308. The analysis was made at a constant flow-rate of 5 μl/min using HBS buffer. The sensor surface had been pre-derivatised with RAM Fc (see Fig. 1). Reagents were introduced in the sequence indicated, as described in Materials and Methods. The differential between readings at the beginning and steady state conditions at the end of each injection pulse is indicated by the dashed line, and is quantitated as a relative response in the report table. Note the failure of MAb 204 to bind in the presence of MAb 308, suggestive that these antibodies share a related epitope.

308, suggesting that these antibodies may recognise a related epitope. However in a similar study on the epitope mapping of HIV-1 p24 [25], several instances were found where the sequence MAb1-p24-MAb2 failed to form a ternary complex, whilst the reversed sequence MAb2-p24-MAb1 allowed the formation of the ternary complex. Such discrepancies might arise from conformationally induced changes in the antigen on binding to the primary antibody. Hence when a negative response is obtained for one pair of antibodies they must be tested in the reverse order, and only when both tests are negative should binding to a similar (or overlapping) epitope be assumed. In the experiments reported herein no examples of false negative responses were observed. When reverse order binding was tested for MAb 308 and 204 (Fig. 4) the results confirmed the original observation. The remaining antibodies tested in Fig. 2 (MAbs 202, 316 and 207) were all clearly capable of binding to hG-CSF after MAb 308, and the antibodies which preceeded them in the sequential injection program. When there is positive binding it can be assumed that antibodies are binding to non-overlapping sites, and it is not necessary to test them in the reverse order. It can be seen that by using a multi-site analysis many relationships can be established in a single run (using four sequential antibodies up to ten relationships, plus the corresponding reciprocal pairs, could be assigned per cycle). The run time was typically 40 min.

Data from another multi-site analysis is shown in Fig 3. In this case, whilst the first three antibodies tested (MAbs 316, 349 and 351) clearly bound to hG-CSF in the presence of MAb 310 showing they have unrelated epitopes, the final antibody tested (MAb 208) did not bind. It cannot be concluded that MAb 208 shares overlapping epitopes with any of the preceeding antibodies in the injection sequence, since failure to bind when any of the preceeding antibodies have already bound to the hG-CSF may be due to steric hindrance by several monoclonal antibodies. Negative responses are therefore only significant when observed directly following the binding of the ligand (note that MAb 208 was not included in the full analysis of the panel of fifteen antibodies presented herein because of insufficient reagent).



Time (s)	AbsResp	RelResp	Id	Time (s)	AbsResp	RelResp	Id
47.0	20788	0	baseline	1567.0	25159	653	349
413.0	22036	1250	310	1826.0	26049	890	351
792.0	23752	1717	anti-AFP	2086.0	26073	24	208
1050.0	23586	103	G-CSF	2346.0	20791	-5282	Reg.
1309.0	24506	650	316				

Fig. 3. Sensorgram of a multideterminant binding analysis using MAb 310. Experimental conditions as for Fig. 2. In this case clear positive binding is observed for all antibodies except 208. However in this example, because a number of antibodies had bound to the G-CSF in the presence of the primary antibody, MAb 310, negative binding may be solely a function of steric hindrance, and is therefore not indicative that MAb 208 has any relationship to any of the other antibodies in this injection sequence.

The results obtained for the epitope mapping of 15 anti-G-CSF MAbs using the biosensor is shown as a reactivity pattern matrix in Fig 4. Analysing these data it was determined that 9 of the antibodies fell into three distinct groups

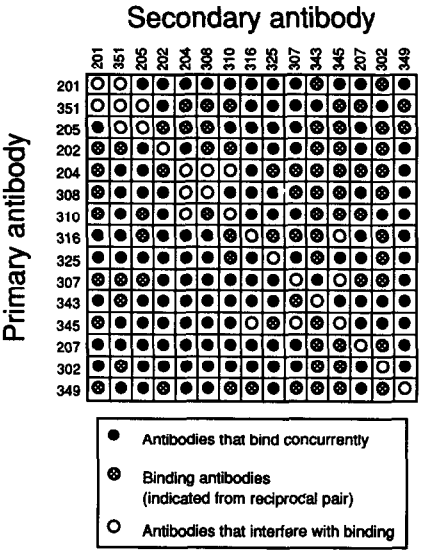


Fig. 4. The MAb reactivity matrix obtained for the Biosensor data. The data obtained from the multideterminant analyses for different MAbs bound to G-CSF is presented as a reactivity matrix in which values of less than 150 RU are scored as interfering antibodies (*i.e.* sharing a related epitope) and values above 150 RU are scored as positive binding (*i.e.* non-related).

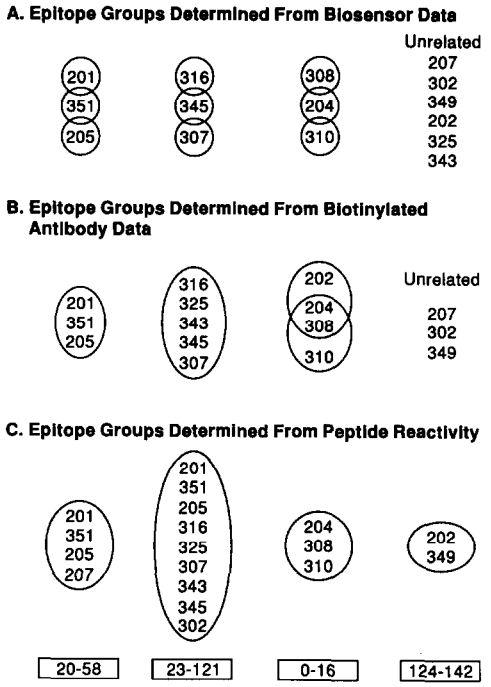


Fig. 5. A comparison of the epitope groups of anti-G-CSF monoclonal antibodies determined by ELISA competition studies or the biosensor.

whilst the remaining 6 antibodies appeared to have unique epitopes (Fig. 5A). Whilst previous studies have shown the use of the biosensor for epitope mapping [21,25], and have indicated good agreement with other data reported in the literature, to date no direct correlation between methodologies, using the same reagents, has been performed. We have previously mapped the regions of hG-CSF recognised by different antibody groups, using competitive ELISAs, as part of a study to identify a functional domain of hG-CSF recognised by neutralising antibodies [12]. Therefore, we can make direct comparisons between these methodologies.

In our previous study the antibodies were firstly screened for their ability to bind reduced and pyridine ethylated hG-CSF and cG-CSF, which shares 80% homology with hG-CSF [26], as well as their ability to neutralise the proliferative response of NFS-60 cells to hG-CSF. It can be seen (Table II and Fig. 5A) that the biosensor classified three of the four neutralising antibodies as being related. Antibody 207, which is the neutralising antibody which does not appear to

TABLE II

BINDING AND NEUTRALISATION CHARACTERISTICS OF ANTI-G-CSF MONOCLONAL ANTIBODIES

Antibodies were tested for neutralisation of the proliferative response of NFS-60 cells to hG-CSF and for binding to reduced, pyridine ethylated hG-CSF (red G-CSF) or canine G-CSF (cG-CSF) in an ELISA. For further details see Layton *et al.* [12].

Antibody	Neutralisation	Binding red G-CSF	Binding cG-CSF
201	+	–	–
351	+	–	–
205	+	–	–
316	–	+	–
345	–	–	+
307	–	–	+
308	–	+	–
204	–	–	+
310	–	–	–
207	+	+	+
302	–	–	–
349	–	+	–
202	–	+	+
325	–	+	+
343	–	+	+

be related, differs from the other neutralising antibodies insomuch as it binds to cG-CSF, and is not dependent on the conformation of G-CSF. Furthermore, additional studies on this antibody using peptide fragments (see below, Fig 5C) showed that MAb 207 bound to peptide 20–58 but was unable to bind to peptide 23–121, unlike the other neutralising antibodies which recognised both these peptides. Thus MAb 207 is unique among the neutralising antibodies in requiring residues 20–22 for binding.

Binding to pyridine ethylated hG-CSF or cG-CSF does not *per se* allow discrimination between groups since some antibodies in all but the neutralising group recognised by the biosensor (MAb 210, 205, and 351) fall into this category (Table II), but it did allow further discrimination between groups in our previous study [12].

The antibodies were categorised further by cross-competition studies. The antibodies were biotin conjugated, and the binding of these antibodies tested in the presence of the 15 unconjugated antibodies which had been used in

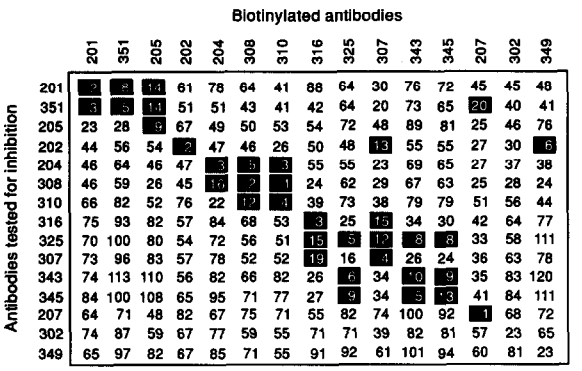


Fig. 6. The reactivity matrix obtained from cross-competition studies with biotinylated monoclonal antibodies to G-CSF. The binding of biotinylated monoclonal antibodies to recombinant hG-CSF in the presence of unlabelled antibody (100 μg/ml) is expressed as the percentage of the binding obtained in the presence of an unrelated control antibody. Combinations giving strong inhibition (0–20% binding) are shaded.

the biosensor study. This data is presented as a reactivity pattern matrix (Fig. 6) for comparison with the analogous data obtained with the biosensor (Fig. 4). The antibody cross-competition data was analysed using the "EPITOPES" program [27]. This program compares the inhibiting antibodies in pairs, calculating a concordance index (CI) and a discordance index (DI), as well as a value for the amount of information (data) for each pair. Antibodies were listed by the program as related if CI > 0.33 and CI > DI [27]. However, because the ELISA competition assay was set up with a high concentration of inhibitory antibody (100 μg/ml) [12] a high level of cross-reactivity between antibody pairs was detected. In most cases the level of binding was less than 100% (Fig. 6) and therefore we defined related groups by including only antibodies with a CI of >0.7. Again three groups were clearly evident (Fig. 5B). It can be seen that there is good agreement between the biosensor data, and that obtained with the biotinylated antibodies, although the correlation is not absolute [for example MAb 343 appears unrelated to the other monoclonal antibodies in the biosensor evaluation (see Fig. 5A)]. One possible explanation for the observed differences could be the effect of the biotinylation on antibody reactivity. Direct inspection of the data (Fig. 6) shows that in some cases of cross-competion with the homologous antibody (*e.g.* MAbs 302 and 349) there is only intermediate inhibition of binding (23% binding). However, the use of the EPITOPES program, which compares data from pairs of *non* conjugated antibodies tends to minimise this effect. In the case of the biosensor methodology no derivatisation of reagents is neccessary, and cross-competition of homologous antibodies was always observed (Fig. 4).

Another possible explanation is that for each method an arbitrary level of inhibition had to be chosen for the delineation of positive versus negative effects, and these arbitary cut-off points may not be exactly equivalent for the two techniques. In addition the 'EPITOPES' program [27] considers intermediate inhibition (21–50% binding) as well as strong inhibition (0–20% binding) whereas the biosensor data was analysed by considering only strong inhibition (*i.e.* total lack of binding as defined by RU values of less than 150.

To define the epitopes further, peptide fragments were generated from recombinant hG-CSF (without reduction, *i.e.* disulphide bonds intact) by chemical or enzymatic fragmentation or by chemical synthesis [12]. Peptides were purified by RP-HPLC and characterised by N-terminal sequence analysis, amino acid analysis and/or plasma desorption mass spectrometry (PDMS) (Fig. 7). The peptides generated were tested for antibody reactivity using an ELISA in which the peptides were coated onto the microtiter plate (at a concentration of approximately 10 pg/well) [12]. The peptides recognised by the antibodies tested on the biosensor are shown in Fig. 5C. The antibodies can be assigned to four groups based on this data as follows: group 1 —those which recognise a region associated with the first disulphide bonded loop of hG-CSF (residues 20–58 including the disulphide bond between C36–C42). This region was identified from a peptide obtained by V8 protease digestion (residues 34–46, eluting at 20.6 min in Fig. 7) and also from synthetic peptides covering this region; group 2 —those which recognise an internal fragment comprising residues 23–121, obtained by cyanogen bromide cleavage of a methionine containing tryptic peptide, residues

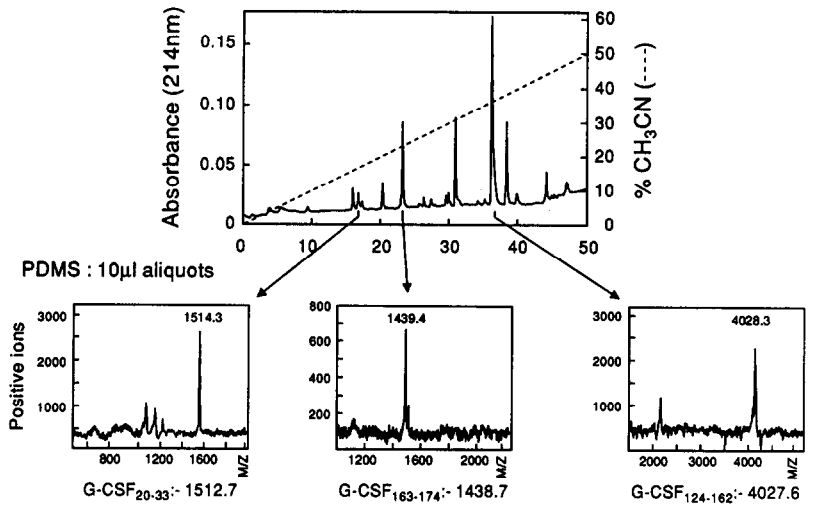


Fig. 7. RP-HPLC separation of the peptides from a *Staphylococcus aureus* V8 digest of recombinant hG-CSF. Recombinant hG-CSF (8 µg) was digested overnight with *S. aureus* V8 protease (enzyme/substrate ratio 1:10) at 37°C in 1% ammonium hydrogencarbonate (100 µl). The resultant peptides were separated by RP-HPLC, using a Pharmacia µRPC PC 3.2/30 column fitted in a Pharamacia SMART system, with a linear 60-min gradient between 0.15% (v/v) aqueous trifluoroacetic acid and acetonitrile–water (60:40) containing 0.125% (v/v/v) trifluoroacetic acid at a flow-rate of 240 µl/min. The operating temperature was 25°C. Fractions were collected automatically at 1-min intervals. Aliquots were taken for plasma desorption mass spectrometry (BioIon 20, Applied Biosystems, CA, USA) and/or N-terminal sequence analysis. Representative mass spectra of the indicated peaks are shown.

23–147; group 3 —those which recognise the N-terminal region comprising residues 0–16 characterised using a tryptic peptide (the recombinant hG-CSF contains an additional methionine residue prior to the authentic N-terminal animo acid which is defined as position 0); group 4 —those which recognise a region towards the C-terminus of hG-CSF (residues 124–142) identified from a V8 protease fragment eluting at 26.6 min (Fig. 7).

Again it can be seen (Fig. 5) that there is excellent correlation between the localisation of the epitopes using the peptide fragments and the epitope groups determined from the biosensor data. Thus, MAbs 201, 351 and 205 comprise part of the group of neutralising antibodies recognising residues 20–58, MAbs 204, 308 and 310 recognise the N-terminal residues 0–16, and MAbs 316, 345 and 307 comprise part of the group recognising the larger internal fragment (residues 23–121). With the exception of the unrelated neutralising MAb 207 (see discussion above) and MAbs 202 and 349 which recognise the C-terminal fragment 124–142, the remainder of the apparently unrelated antibodies also recognise residues in the larger internal fragment. The biotinylated antibody data also found MAb 349 to be unrelated, although MAb 202 appeared to be related to the antibodies recognising the N-terminal fragment (group 3). However, if only the strongest level of inhibition is considered for the cross-competition data (boxed numbers in Fig 6), the data suggest that MAb 202 is related to MAb 349 and 307 is not closely related to MAbs 204, 308 and 310. Thus, in this case, the inclusion of the intermediate inhibition data from the biotinylated antibody studies may have given a misleading result in the case of MAb 202.

CONCLUSIONS

We have presented data on the epitope mapping of 15 monoclonal antibodies raised against recombinant hG-CSF. This data was obtained using an instrumental biosensor employing the optical detection system of surface plasmon resonance. A multideterimant assay was used by the formation of molecular complexes around the ligand. The binding specificity of a number of

antibodies could be analysed in a single assay. The method is rapid and does not require the derivatisation of the monoclonal antibodies or G-CSF. Excellent agreement was obtained between the epitope groups determined using the biosensor, and similar studies using ELISA cross-competition with biotinylated antibodies or antibody binding to peptide fragments [12].

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