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Reversed-phase high-performance liquid chromatographic tryptic digest peptide map comparisons of monoclonal antibodies to human tumor necrosis factor

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ABSTRACT

An automatic computer technique was used to compare the retention times and ultraviolet spectra of sixty-two peaks in peptide maps of three monoclonal antibodies against human tumor necrosis factor (TNF) and one monoclonal antibody against recombinant factor VIII. The anti-TNF monoclonal, B6, which has an overlapping epitope with the anti-TNF monoclonal, A10G10, had a 90% peak match with A10G10. The anti-TNF monoclonal, A6, with a different epitope to TNF than A10G10, had only a 60% peak match. The A6 match to A10G10 was similar to the 50% peak match of the anti-factor VIII monoclonal with A10G10. The results of this study suggest that peptide mapping can be used as a quantitative characterization technique for comparing monoclonal antibodies.

INTRODUCTION

It is frequently of interest to distinguish among monoclonal antibodies to the same antigen. Characterization is usually achieved with immunological studies comparing avidities and epitopes; however, these techniques may lead to ambiguous results such as the inability to determine whether two antibodies have the same or overlapping epitopes. In this study, peptide mapping of typsin digests using reversed-phase high-performance liquid chromatography (HPLC) was evaluated as a characterization technique. This technique has become a widely accepted analytical method for monitoring the primary sequence of proteins produced by recombinant DNA technology [1,2]. Tryptic fragments of extensively denatured proteins are obtained from both the surface and interior of the protein and vary in size from approximately two to twenty amino acid residues in length. Using this technique, proteins with different sequences would be expected to produce peptides that differ in hydrophobicity and thus differ in retention time. In addition, differences in the amino acid sequence near the lysine and arginine residues may effect the hydrolysis rate of trypsin and therefore the amount or chromatographic peak height/area of the peptides. As an example of the power of the technique, the substitution of a glutamic acid residue for an arginine in recombinant tissue-type plasminogen activator has been shown to be easily detected [1].

The purpose of the present study was: (1) to evaluate trypsin digest reversed-phase HPLC mapping as a method to discriminate between monoclonal antibodies found by immunological techniques to have similar avidities and the same or overlapping epitopes for tumor necrosis factor (TNF); and (2) to evaluate an automatic computer peak retention time and ultraviolet spectra library search technique as a means of objectively and quantitatively comparing complex monoclonal antibody trypsin digest maps.

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EXPERIMENTAL

Reagents

Acetonitrile (UV grade), trifluoroacetic acid and high-purity water were all Burdick and Jackson brand. Sequencing-grade trypsin was obtained from Boehringer Mannheim (Indianapolis, IN, USA). Bovine serum albumin (BSA) was obtained from Miles Pentex (Kankakee, IL, USA). Tetramethyl benzidine (TMB) was obtained from Kirkegaard & Perry Labs. (Gaithersburg, MD, USA). Highly purified ($2–4 \cdot 10^8$ U/mg) recombinant human TNF was obtained from Chiron (Emeryville, CA, USA). Biotin X-NHS was obtained from Calbiochem-Behring (La Jolla, CA, USA). Goat anti-mouse immunoglobulin G (IgG) biotin and Streptavidin-HRP conjugate were obtained from Zymed (San Francisco, CA, USA). The IgG_1 anti-TNF monoclonal antibodies A10G10, A6 and B6 were purified from tissue culture fluid by polyethylene glycol precipitation, two back to back anion-exchange chromatography steps and finally by size-exclusion chromatography. The IgG_1 anti-coagulation factor VIII monoclonal antibody, C7F7, was purified from tissue culture fluid by Protein A chromatography [3]. Each purified unreduced monoclonal antibody appeared as one band by the technique of silver-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [4]. The molecular mass of the monoclonal antibodies was assumed to be 160 000 Da. The protein concentrations of the antibodies were calculated from the absorbances at 280 nm using a molar absorptivity of 13.8 for a 1% solution at 280 nm.

Immunological methods

The enzyme-linked immunosorbent assay (ELISA) used to determine antibody binding to TNF was performed as follows: microtiter plates were coated with TNF (0.4 μg/ml) in 15 μg/ml BSA–phosphate buffered saline, pH 7.2 (PBS) and were incubated overnight at 4°C. The coating technique for this assay was somewhat unusual in that a small amount of BSA was included in the coating buffer to minimize the amount of TNF required to obtain a maximal signal-to-noise ratio [5]. The plates were blocked with 1% BSA, 0.05% Tween 20, PBS for 1 h at 37°C. The anti-TNF monoclonal antibody samples were added in serial dilutions in the blocking buffer. Bound anti-TNF was detected with anti-mouse IgG-biotin followed by streptavidin-HRP and TMB substrate. The plates were washed between all steps with 0.05% Tween 20, PBS. All detection reagents (except TMB) were diluted in the blocking buffer and incubations were done at 37°C for 1 h. The intensity of yellow color generated in the assay was proportional to the amount of peroxidase (HRP)-labeled streptavidin bound and was determined by measuring the absorbance at 450 nm on a Dynatech MR600 microtiter plate reader (Dynatech, Burlington, MA, USA). The reading for each well was compensated for non-specific contributions by dividing by the absorbance at 570 nm. Experiments were done in duplicate on the same ELISA plate and average values utilized in the data analysis method. Binding curves of the different monoclonals were compared by normalizing the data. The highest average absorbance value for each monoclonal was assigned a value of 1 and all other values were calculated to be fractions of this average absorbance maximum.

The competition experiments followed the same coating and blocking steps as the anti-TNF binding assay. A mixture of varying amounts of an antibody to TNF and a fixed amount of A10G10-biotin (biotinylated according to the Calbiochem-Behring method supplied with the reagent) was then added to the plates. The plates were washed between all steps with 0.05% Tween, PBS. Bound A10G10-biotin was detected with streptavidin-HRP and TMB substrate. The data were analyzed as described above.

Trypsin digestion of monoclonal antibodies

A 200-μl volume of approximately a 20 mg/ml antibody solution was added to 1.8 ml of 6 *M* guanidine hydrochloride–0.5 *M* Tris, pH 8.6. A 50-μl volume of 2 *M* dithiothreitol was added and the sample was heated at 100°C for 1 h. A 60-μl volume of 2 *M* iodoacetic acid was added and the sample was incubated in the dark for 30 min. The samples were then dialyzed extensively against 0.1 *M* ammonium bicarbonate. The samples were digested by adding 4 μg of trypsin to

400 μg of antibody (trypsin/antibody molar ratio of 0.056) and incubating at 37°C for 18 h. The digests were aliquoted and stored at −70°C.

High-performance liquid chromatography

Of each sample 200 μl (400 μg) were analyzed by reversed-phase HPLC using an HP 1090 Series M system with an integrated diode-array detector. HP 79994A ChemStation software was used for data analysis. A Vydac 218TP54 (C_{18}, 5μm, 250 mm × 4.6 mm I.D.) column was used with the following chromatographic conditions: flow-rate, 0.7 ml/min; oven temperature, 40°C; detection, 220 nm; solvent A, 0.1% TFA; solvent B, 0.1% TFA–60% acetonitrile; gradient, 0 min 0% B, 5 min 0% B, 120 min 70% B, 125 min 100% B, 130 min at 1.5 ml/min 0% B, 155 min 0% B. The wash with 100% B (0.1% TFA–60% acetonitrile) was used to wash the column.

Peptide mapping data analysis

Peptide maps of the three anti-TNF monoclonals and one anti-factor VIII monoclonal were obtained in duplicate using freshly prepared chromatography reagents. Spectra of peaks with a 10.0 mA.U. threshold and 0.100 min peak width were collected in the peak-controlled mode. In the peak-controlled mode the diode-array detector automatically obtains the spectra from 210 to 400 nm at the upslope, apex and downslope of each peak that meets the threshold and peak width criteria. The areas of more than 100 peaks contained in the run 1 220-nm chromatogram of the A10G10 monoclonal antibody were automatically determined using the ChemStation software peak integrator function with the default baseline settings (0.300 min peak width, 0 units (4.0 mA.U.) threshold, 1 min × mA.U. area reject). Each of the integrated peaks was then analyzed automatically for peak purity using the "softkey" Peak Purity in the Data Editor of the ChemStation software. Peak purity or peak homogeneity is determined by this software program by automatically normalizing and comparing the peak spectra collected at the upslope, apex and downslope [6,7]. A numerical point by point comparison of the UV spectra is implemented on the ChemStation and peak purity scores are calculated. At the extremes, a score of 0 indicates no match while 1000 indicates identical spectra. Values above 990 indicate that the spectra at the upslope, apex and downslope of the peak are similar. Seventy-nine peaks had a peak purity score of greater than 990 by this method of analysis, and the spectra and retention times of these peaks were entered into a Data Editor Library. The retention times and spectra for each peak in the 220-nm chromatograms of the monoclonal antibodies were then compared to the library of A10G10 peak retention times and spectra using the automated Library Search Macro (rev. 4.06) with a match threshold of 995, no reference spectrum or smoothing, a time window of 2.0% (retention time plus and minus 1%) and a peak purity threshold of 990.

RESULTS

Anti-TNF binding and competition

The TNF binding curves for the three TNF monoclonal antibodies A10G10, B6 and A6 are shown in Fig. 1. The curves are very similar suggesting that the antibodies bind to immobilized TNF with similar avidities. To compare the epitopes of the three monoclonal antibodies competition experiments were performed (Fig. 2). In this assay, if the unlabeled and labeled antibodies bind to overlapping sites on the TNF molecule, the amount of labeled antibody bound to the immobilized TNF will be reduced [8]. A reduction in the amount of labeled antibody bound to TNF was apparent in the control experiment of unlabeled A10G10 competing with labeled A10G10

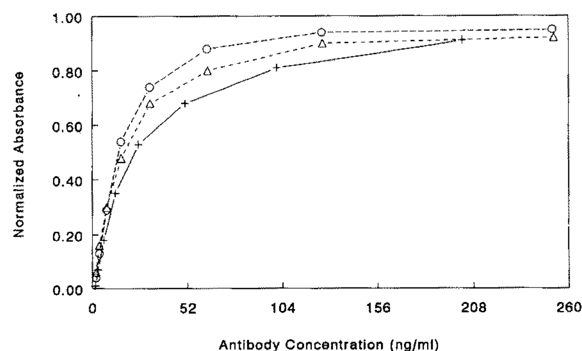

Fig. 1. ELISA binding curves of three monoclonal antibodies, designated as A6 (+), A10G10 (△) and B6 (○), to human tumor necrosis factor immobilized on a microtiter plate.

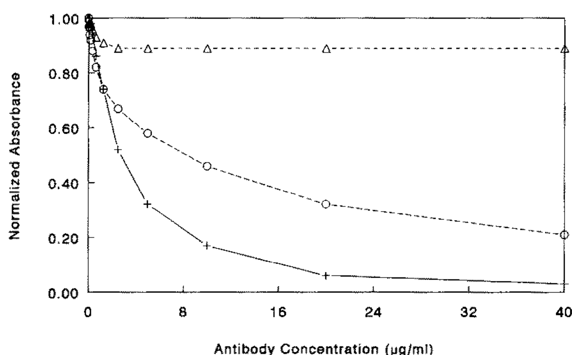

Fig. 2. ELISA binding curves showing competition of A10G10 (+), A6 (△) and B6 (○) with A10G10-biotin for binding to human tumor necrosis factor immobilized on a microtiter plate.

and in the partial competition of A10G10 with B6. These results suggest that A10G10 and B6 have the same, similar, or overlapping epitopes. On the other hand, there was little or no competition of A10G10 with A6 for binding to TNF suggesting that the two monoclonals have different epitopes.

Peptide mapping

A mirror image comparison of the peptide maps of the two different runs of the A10G10 trypsin digest are shown in Fig. 3. The retention times, peak heights and areas of the two chromatograms appear to be identical. Mirror image comparisons of the trypsin digests of B6 and A6 with A10G10 are shown in Figs. 4 and 5, respectively. The B6 map is very similar to that of A10G10 and the A6 map is not. As a control, a mirror image comparison of the trypsin digest of

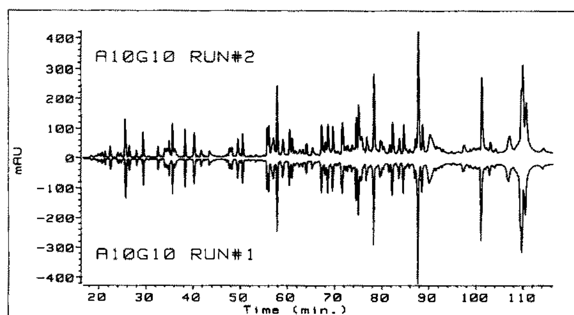

Fig. 3. Mirror image comparison of trypsin digest peptide map chromatograms at 220 nm of A10G10 obtained on two different days with fresh chromatography reagents.

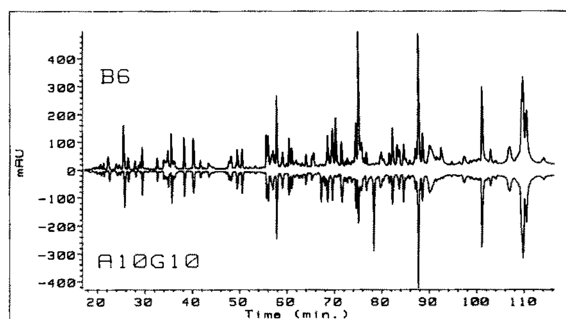

Fig. 4. Mirror image comparison of trypsin digest peptide map chromatograms at 220 nm of B6 and A10G10.

A10G10 with C7F7, a monoclonal antibody that recognizes a different antigen than A10G10, is shown in Fig. 6. Although different, the maps have a number of peaks in common.

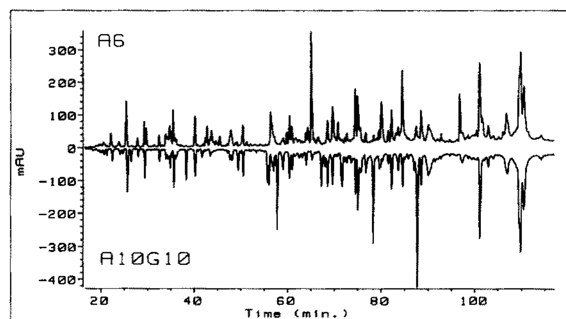

Fig. 5. Mirror image comparison of trypsin digest peptide map chromatograms at 220 nm of A6 and A10G10.

In order to obtain a quantitative and objective comparison of the maps for the four antibodies, a library of the retention times and spectra of 220-nm peaks from the first chromatographic run of A10G10 with peak purity scores greater than 990

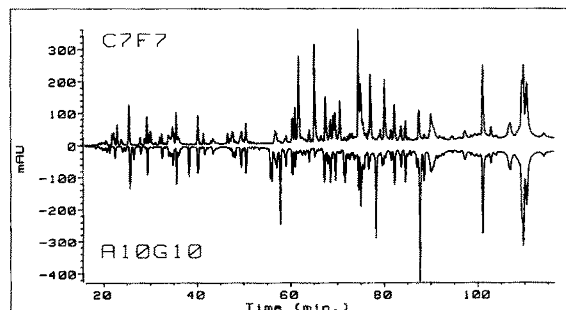

Fig. 6. Mirror image comparison of trypsin digest peptide map chromatograms at 220 nm of C7G7 and A10G10.

was generated. Using the computer software supplied with the HPLC instrument, the retention times and spectra of the peaks in the peptide maps of the other monoclonals were compared to the A10G10 library. Seventy-nine A10G10 peaks from run 1 were entered into the library. As a check, A10G10 run 1 was then compared to the library and there were 78 peak matches. A comparison of A10G10 run 2 with the library was then undertaken and 63 peak matches were found. This difference between the two runs (which qualititatively appear to be identical, Fig. 3) is probably due to subtle differences in chromatographic conditions resulting in differences in recognition by the software of very small peaks near the threshold of detection [6]. Sixty-two peaks were found to be reproducible in the two runs of A10G10. Two runs each of A6, B6 and C7F7 were then automatically evaluated by the library matching program using the 62 reproducible A10G10 peaks. For B6, 55 peaks in run 1 and 54 peaks in run 2 were matched to the A10G10 library for an average match of 87.9%. For A6, 37 peaks in run 1 and 38 peaks in run 2 were matched to A10G10 for an average match of 60.5%. For C7F7, 34 peaks and 33 peaks for runs 1 and 2, respectively, were matched to A10G10 for an average match of 54.0%.

DISCUSSION

One of the principle aims of this study was to evaluate reversed-phase HPLC peptide mapping as a supplementary characterization method to discriminate between similar monoclonal antibodies. Unambiguous discrimination between monoclonal antibodies to the same antigen is frequently not possible using immunological techniques. This is best illustrated by the two anti-TNF monoclonals A10G10 and B6 in this study which bind with similar avidities to TNF and have the same, similar, or overlapping epitopes. However, their peptide maps were distinctly different. Although a simple qualitative inspection of the maps of the monoclonals can be used to evaluate differences, a more quantitative approach was desired. Quantitation by visual "mirror image" comparison of retention times and peak areas to determine peak matches in complex maps, such as those resulting from trypsin digests of monoclonal antibodies, is quite laborious and to some extent subjective in nature. An automatic technique using computer software supplied with the HPLC system to compare retention times and spectra of the peptide peaks was found to give both quantitative and objective comparisons of the maps. Peaks found to match by this method do not necessarily contain identical peptides as only limited spectral data can be obtained from peptides that lack aromatic residues. In addition, the method is limited by run-to-run differences in baselines and inconsistent computer integration of small peaks near the threshold of detection [6,9].

IgG molecules are composed of two identical heavy-chain polypeptides of approximately 55 000 Da and two identical light chains of approximately 25 000 Da. The light chains are approximately 220 amino acids long and can be divided into two regions of approximately 110 amino acids in length: an amino-terminal half with a heterogeneous amino acid sequence (variable region) and the carboxy-terminal half with essentially the same amino acid sequence (constant region). In addition, there are two types of light-chain constant regions, κ and λ, classified according to structural similarities. The TNF monoclonal antibodies used in this study all had κ light chains. The C7F7 light-chain type was not determined. The IgG heavy chains are approximately 440 amino acids long and are divided into four regions that contain approximately 110 amino acids: one region is variable and three are constant. The heavy chains can be divided into four subclasses based on sequence homologies and all four monoclonals in this study belong to the IgG_1 subclass. The variable regions of one heavy chain and one light chain combine to form one antigen binding site [10–13].

Approximately 50% of the peptide peaks in the peptide map of the factor VIII monoclonal antibody were found to match up with those contained in the A10G10 TNF monoclonal antibody library. These results suggest that the matching peptides are located in the IgG_1 heavy- and light-chain constant regions. The TNF monoclonal antibody, A6, which was found by immunological techniques to have a different TNF epitope

than A10G10, had only a 60% match with A10G10. These results suggest that the variable regions of the two monoclonals are extensively different as might be expected. The constant regions of the two monoclonals would be expected to be the same because they have κ light chains and γ_1 heavy chains. On the other hand, B6, which has the same or an overlapping TNF epitope with A10G10, matched 90% with A10G10. These results suggest that B6 and A10G10 have slightly different variable regions and that they have overlapping rather than identical epitopes.

The results of this study indicate that reversed-phase HPLC tryptic digest peptide mapping can be used as a quantitative tool to supplement more traditional immunological methods for the characterization of monoclonal antibodies.

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