Pharmaceutical Research, Vol. 9, No. 11, 1992

Research Article

Identification of Sites of Degradation in a Therapeutic Monoclonal Antibody by Peptide Mapping

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Received April 1, 1992; accepted June 23, 1992

A peptide mapping procedure was developed to locate regions of a monoclonal antibody, OKT3, that undergo chemical modification as the molecule degrades upon storage. The structures of these peptide degradation products were investigated. Deamidation at specific asparagine residues and oxidation of a cysteine and several methionines were found to be major routes of OKT3 degradation. A unique chain cross-linked degradation product was also observed and characterized. Changing the storage conditions of the antibody affected the relative distribution of degradation products. These results were useful in the development of more stable formulations for OKT3, and the methods can be used in the characterization of other monoclonal antibodies intended for therapeutic use.

KEY WORDS: OKT3, monoclonal antibody; peptide mapping; protein stability; degradation of proteins.

INTRODUCTION

Orthoclone OKT3 became the first monoclonal antibody approved for therapeutic use in 1986. OKT3 is used clinically to reverse rejections of human kidney transplants and is being studied as an adjunct therapy in other organ transplants. This murine antibody derives its immunosuppressive properties from being directed against a component of the human T-cell antigen receptor complex. When OKT3 binds to the receptor, T-cell function is blocked and peripheral T cells are removed from circulation, halting the patients' immune response to the transplanted tissue.

The production of proteins for pharmaceutical purposes requires extensive characterization of the purity, biochemical properties, and stability of the protein. Proteins have an inherent propensity to undergo a variety of chemical reactions that can lead to loss of structural integrity and bioactivity (for a review of stability of proteins, see Ref. 1). The effect of storage conditions and formulation on the protein drug candidate needs to be investigated to minimize formation of damaged protein. As a marketed therapeutic, considerable effort has been extended in developing methods to analyze OKT3 and monitor the antibody's stability. With a molecular weight of about 150,000, OKT3 is a relatively large protein and its size makes it extremely difficult to characterize fully its degradation products.

In stability studies of OKT3, several analytical assays indicated that structural changes occurred in some batches of the product within 1 year of storage at 5°C and after a few months at 24 and 37°C. These assays include isoelectric focusing, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE), ion-exchange HPLC, and gel permeation chromatography. These assays measure different physical properties, and the observed changes are a reflection of the multiple modes of degradation that would be available to a protein molecule the size of OKT3. The results of additional studies of the stability of the antibody suggested that three major mechanisms account for most of the OKT3 decomposition: oxidation of labile amino acids, deamidation of Asn or Gln residues, and fragmentation of peptide chains (2).

This paper reports the use of a peptide mapping procedure to identify specific sites and chemical mechanisms of degradation in OKT3. The effect of different storage conditions on these degradative pathways was investigated. This information can be applied to optimizing production methods and formulations for OKT3 to preserve molecular integrity. Also, since the major portion of the structure of antibodies is the constant region, in which the amino acid sequence is conserved in antibodies of the same class, information on sites of OKT3 degradation will be useful in the development of other antibodies as therapeutic agents, as many of the same degradation pathways would be expected to occur.

MATERIALS AND METHODS

OKT3 Antibody. Production lots of Orthoclone OKT3 were used as a source of antibody for these studies. This material was formulated at 1 mg/ml sterile solution in pH 7.0 phosphate-buffered saline containing polysorbate 80. Vials containing 5 ml of OKT3 solution were stored in a refrigerator or in stability cabinets at elevated temperatures. For peptide mapping, the antibody was concentrated with a Centricon 30 apparatus before use. The integrity of the antibody

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0724-8741/92/1100-1386$06.50/0

was checked on a Pharmacia PhastSystem electrophoresis unit by isoelectric focusing on pH 3–9 Phastgel IEF media and SDS/PAGE on 10–15% Phastgels, with and without reduction of the sample by heating with 2-mercaptoethanol. The gels were stained with Coomassie Blue. Biological activity of the antibody was assessed by measuring binding to the antigen on T cells (the Potency Assay described in Ref. 2); this assay was performed by the Ortho Biotech QA department or the Bioanalytical Research group of R. W. Johnson Pharmaceutical Research Institute.

Separation of OKT3 H and L Chains. Disodium 2-nitro-5-thiosulfobenzoate (NTSB) was synthesized by the literature procedure (3). The sulfitolysis reagent was prepared by adding 0.40 ml of NTSB solution to 4.6 ml of a solution of 4.73 g of guanidine thiocyanate, 0.504 g of sodium sulfite, 20 mg of EDTA, and 0.485 g of Tris hydrochloride in water. The pH of the solution was adjusted to 9.0 with KOH. To 1 ml of OKT3 solution was added 1 ml of sulfitolysis reagent and the reaction allowed to proceed for 45 min. Portions of the reaction mixture were loaded onto a Pharmacia HR10/30 Superose 12 column and the column was eluted with phosphate-buffered saline containing 6 *M* urea on a FPLC system. Fractions containing pure heavy (H) and light (L) chain, as determined by SDS/PAGE, were used for peptide mapping. Material containing the 88-kD component was obtained as an incompletely separated small peak eluting in front of the H chain.

Peptide Mapping. The H and L chain fractions were concentrated to 0.2 vol with a Centricon microconcentrator, then diluted with an equivalent volume of 50 m*M* Tris, pH 8.5, buffer for trypsin digestion or 100 m*M* dibasic sodium phosphate for Glu-C endoproteinase digestion. To the substrate solution was added sequence grade enzyme (Boehringer-Mannheim) in an enzyme to substrate ratio of approximately 1:50; the enzyme was added in two portions 4–5 hr apart. The digestion mixtures were incubated at 25°C for 20 hr, then quenched by the addition of trifluoroacetic acid to 10% volume. The digests were analyzed by reversed-phase HPLC on a 4.6 × 250-mm, 5-μm Rainin Dynamax C18, 300A column eluted with gradients of increasing acetonitrile in 0.1% trifluoroacetic acid (TFA) on a Perkin–Elmer gradient HPLC system. The eluate was monitored at 215 and 280 nm with a diode array detector. Data collection and processing were performed with a Nelson Chromatography Data System on an IBM PS2 computer. Sample chromatograms are shown in Fig. 2.

Characterization of Peptides. Peptide components of the HPLC maps were manually collected and the fractions dried using a Savant Speedvac. The amino acid sequence of the peptides was determined on a Porton 2090E Integrated Micro-Sequencing System or an ABI 477A Protein Sequenator. Sequencing of OKT3 fragments directly from PVDF electroblots of SDS/PAGE gels was accomplished following the procedure described by Matsudaira (4) and loading the blots as instructed in a Porton user bulletin. The molecular weights of the peptides were determined by FAB mass spectrometry on a Finnigan-MAT 8230 mass spectrometer with 8-keV xenon atoms. The identity of some OKT3 peptide fragments was established by running the peptide map on an HPLC with a solvent stream splitter interfaced via a pneumatically assisted electrospray source to a Sciex TAGA 6000E triple-quadruple mass spectrometer at Cornell University. The calculated masses of the peptides were compared with the expected molecular weights of OKT3 tryptic fragments and the peptides were located on the map by simultaneous detection by UV.

Peptide Synthesis. To confirm the identity of several OKT3 peptide fragments, the HPLC retention times were compared with authentic peptides obtained by chemical synthesis. Peptides were synthesized on an ABI 430A Peptide Synthesizer using standard protocols for t-BOC amino acid derivatives. BOC-methionine sulfoxide and BOC-aspartic acid α-benzyl ester were purchased from Bachem Bioscience, Inc. The peptides were cleaved from the resin with HF containing 10% *p*-cresol for 1 hr at 0°C. After removal of the HF with vacuum and washing the residue with ethyl acetate, the peptide was extracted into TFA. The mixture was filtered and the filtrate reduced to small volume by rotary evaporation. The addition of ether to the residue precipitated the crude peptides. The peptides were purified by prep reversed-phase HPLC and identity confirmed by amino acid analysis and FAB mass spectrometry.

Preparation of $F(ab')_2$ Fragments. OKT3, 5 mg/ml, was dialyzed against 0.9% sodium chloride. The antibody was transferred to a polypropylene tube and diluted with 1.5 ml 0.20 *N*, pH 4.1, citrate buffer. To this solution was added 300 μl pepsin solution, 1.0 mg/ml, in 0.1 *N* citrate, pH 4.1. The mixture was incubated at 37°C for 2 hr, then another 150 μl of pepsin solution added and the incubation continued. After an additional 1.5 hr, the digestion was stopped with 0.50 ml 1 *M* Tris. The solution was filtered through a 0.45-μm membrane filter, then concentrated with a Centricon 30 apparatus to less than 2 ml. The protein was loaded onto a 16/50 Pharmacia column of Superose 12 prep grade and eluted with PBS. The center fraction cut of the major peak was concentrated with a Centricon 30 and treated with the sulfitolysis reagent, followed by size exclusion chromatography as described for intact OKT3 above. With the $F(ab')_2$ prepared from reference standard OKT3, SEC gave a single protein peak which contained both the L chain and the H chain $F(ab')_2$ fragment, as shown by peptide mapping comparison with standard L and H chain digests. $F(ab')_2$ from degraded OKT3 had a second, earlier-eluting peak in the SEC which had an apparent molecular weight of 50 kD on SDS/PAGE. This material was digested with trypsin and the peptide map compared with the map of standard OKT3 $F(ab')_2$.

RESULTS AND DISCUSSION

Peptide Mapping. A peptide mapping procedure was developed to locate regions of OKT3 that were chemically altered as the antibody degraded upon storage. Mechanisms of OKT3 degradation were deduced by determining the structures of peptide fragments isolated from the enzyme digests produced in the mapping procedure. OKT3 samples stored under different conditions for various lengths of time were analyzed by this peptide mapping procedure.

Native antibodies are not very susceptible to enzymatic digestion. Denaturation exposes sites for attack by enzymes but has a tendency to make the antibody insoluble. In our method, OKT3 was denatured and the disulfide bonds

cleaved along with Cys *S*-sulfonation following the procedure of Thannhauser *et al.* (3). Cys-sulfonation improves the solubility of the protein and subsequent peptide fragments. This technique also enabled the separation of the H and L chains of OKT3 by size exclusion chromatography. Inclusion of 6 *M* urea in the elution buffer was necessary to prevent the chains from aggregating. The H and L chains were then separately digested with enzymes, which greatly simplified the chromatography needed to separate the more than 60 peptide fragments produced by trypsin digestion of the two chains. The digests were analyzed by reversed-phase HPLC to produce the peptide map. The peptide maps of degraded samples of OKT3 were compared to the maps of reference standard material to detect peptide fragments that had undergone structural changes.

It should be noted that this method discriminated against detecting degradation due to hydrolysis of peptide bonds since the resulting chain fragments may be separated from the H and L chain by the size exclusion chromatography and not be included in the material digested with enzymes for the peptide maps. Decomposition of OKT3 due to chain fragmentation does occur over time, as evidenced by SDS/PAGE, and other methods are being used to study OKT3 degradation pathways of this type.

Several enzymes were investigated as digesting agents for peptide mapping of OKT3; Glu-C and Lys-C endoproteinases produced fewer peaks in the chromatogram than trypsin, but the large size of many of the fragments made complete chemical characterization of the peptides difficult and changes in the structure of one amino acid did not alter the retention times significantly. The more complex chromatograms of the trypsin digests gave more useful information for localizing the sites of OKT3 degradation to specific residues in the amino acid sequence of the molecule. Glu-C and Lys-C digestions were useful in confirming the identity of certain peptides and amino acid modifications.

Peptide fragments of OKT3 were identified by collecting the material eluting as peaks in the peptide map and determining the structure of each peak component by amino acid sequencing, mass spectrometry, and, in some cases, coelution with chemically synthesized peptides. Digestion of OKT3 chains with trypsin was found to result in several incomplete cleavages at Lys–Asp bonds, e.g., L182–183, and alternate cleavages at adjacent basic residues, e.g., H362–363 (see OKT3 amino acid sequence; Fig. 1). Examples of HPLC tryptic peptide maps of OKT3 L and H chains are shown in Fig. 2.

Degradation upon Storage at Elevated Temperature; Asparagine Deamidation. At 37°C, the earliest detectable change in OKT3 is a shift in the pattern of isoelectric focusing (IEF) gel bands toward a more acidic pI; observable changes occur in as little as 1 week (2). Though less sensitive than IEF, ion-exchange HPLC changes in retention time, indicating a more acidic species, are also observed at later time points. Samples held at 5°C, however, continue to exhibit IEF patterns with minimal change from control for 9 months. The change in IEF pattern is not correlated with loss of antigen binding potency of OKT3. The rate of this IEF shift is not affected by the addition of antioxidants or filling the sample vials with an overlay of an inert gas such as nitrogen or argon. It had been proposed that the IEF acidic band shift is due to the deamidation of Asn or Gln residues, as has been observed previously for other proteins (5,6).

LIGHT CHAIN

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  1  QIVLTQSPAI MSASPGEKVT MTCSASSSVS YMNWYQQKSG TSPKRWIYDT
 51  SKLASGVPAH FRGSGSGTSY SLTISGMEAE DAATYYCQQW SSNPFTFGSG
101  TKLEINRADA APTVSIFPPS SEQLTSGGAS VVCFLNNFYP KDINVKWKID
151  GSERQNGVLN SWTDQDSKDS TYSMSSTLTL TKDEYERHNS YTCEATHKTS
201  TSPIVKSFNR NEC
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HEAVY CHAIN

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  1  QVQLQQSGAE LARPGASVKM SCKASGYTFT RYTMHWVKQR PGQGLEWIGY
 51  INPSRGYTNY NQKFKDKATL TTDKSSSTAY MQLSSLTSED SAVYYCARYY
101  DDHYCLDYWG QGTTLTVSSA KTTAPSVYPL APVCGDTTGS SVTLGCLVKG
151  YFPEPVTLTW NSGSLSSGVH TFPAVLQSDL YTLSSSVTVT SSTWPSQSIT
201  CNVAHPASST KVDKKIEPRG PTIKPCPPCK CPAPNLLGGP SVFIFPPKIK
251  DVLMISLSPI VTCVVVDVSE DDPDVQISWF VNNVEVHTAQ TQTHREDYNS
301  TLRVVSALPI QHQDWMSGKE FKCKVNNKDL PAPIERTISK PKGSVRAPQV
351  YVLPPPEEEM TKKQVTLTCM VTDFMPEDIY VEWTNNGKTE LNYKNTEPVL
401  DSDGSYFMYS KLRVEKKNWV ERNSYSCSVV HEGLHNHHTT KSFSRTPGK
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Fig. 1. Amino acid sequence of OKT3. The amino acid sequence of the light and heavy chains of OKT3 in the single-letter code as derived from cDNA sequencing. The underlined portions indicate peptide fragments which we identified as containing sites of degradation by peptide mapping and are referred to in the text. In the mature protein the amino terminus of both chains is cyclized to the pyroglutamic acid derivative, the carboxy terminal Lys of the H chains is processed off, and a carbohydrate chain is attached to Asn H299. OKT3 is an IgG2a isotype.

OKT3 held at 37°C for 2 months showed one major change in the trypsin-generated peptide map of both the L and the H chain. As shown in the L chain map of degraded OKT3 in Fig. 3, compared to the control in Fig. 2, the peak with a retention time (rt) of 26.5 min (peak 1) diminished in size, and two peaks with rt of 27.1 and 27.4 min increased considerably in size (peaks 2 and 3). The peak area ratio of peak 3 to peak 2 was 3:1. Sequencing of these peaks established that the normal tryptic fragment, peak 1, is L155–168 and the degradation peak 2 is the Asp^{156} analogue of this peptide, Asn being at this position in undegraded OKT3. The peak 3 component showed a Gln at the first cycle of the sequencing, then the Edman degradation terminated. This result is consistent with this peptide being the isoaspartate-156 analogue of L155–168, since Edman sequencing fails at isoAsp residues (7) and the other expected product of Asn deamidation is isoAsp (8). Asparagine deamidation via a cyclic succinimide derivative has been shown to give both types of aspartate derivatives at similar product ratios in other peptides and proteins (9–11).

As in the L chain, one major change was observed in the H chain tryptic map of thermally stressed OKT3. A decrease in the size of a peak identified by sequencing as H364–388 was observed and two new nonresolved peaks, eluting slightly earlier, appeared (Fig. 4). The degradation peptides

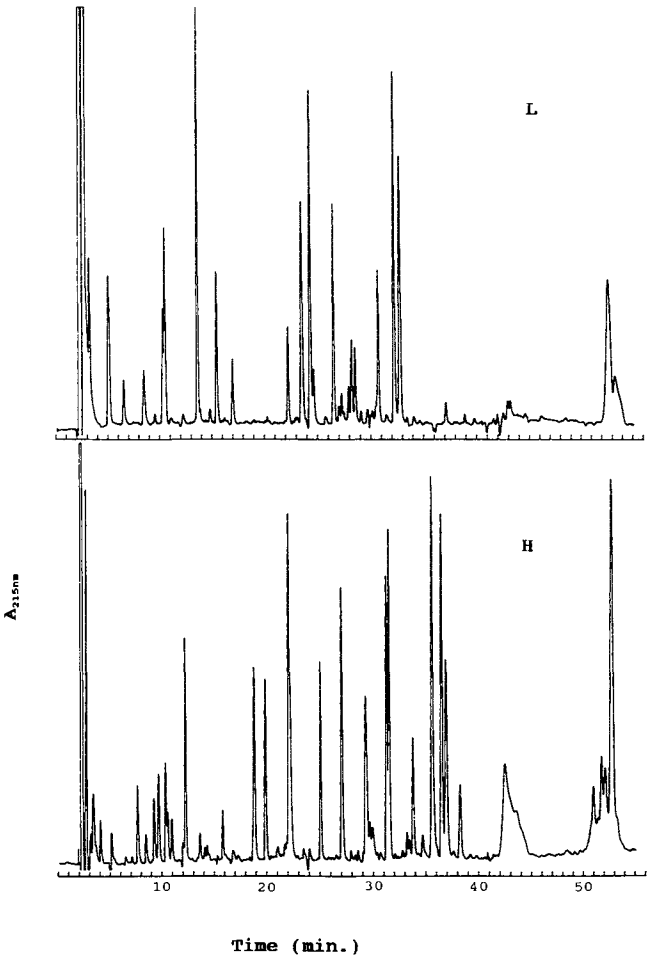

Fig. 2. Peptide maps of OKT3 light and heavy chains. Separated L and H chains from OKT3 reference standard were digested with trypsin and the digests analyzed by HPLC on a Rainin Dynamax C18, 5-μm 300A column eluted with a gradient of 5 to 45% acetonitrile in 0.1% trifluoroacetic acid over 60 min at 1.5 ml/min.

isolated from the combined nonresolved peaks were related to H364–388 by the identical results obtained in sequencing through 22 cycles. This peptide contains two asparagines, which could potentially deamidate. Asn^{385} was observed in the sequencing, but the status of Asn^{386} could not be established due to the low yields at this cycle of the sequencing and carryover from Asn^{385}. This is particularly true since the expected major product of deamidation, the isoAsp peptide, would be diagnosed only as a termination in the sequencing. Asparagine deamidation at H386 in this sample of OKT3 was confirmed by digesting the heavy chain with Glu-C endoproteinase, which gives a small H383–390 fragment. Asn^{386}-, Asp^{386}-, and $isoAsp^{386}$-H383–390 peptides were synthesized and found to coelute with the peaks in the Glu-C maps of heat-treated OKT3 which exhibited the greatest differences in relative peak height from standard. By using a shallow, extended gradient elution on the HPLC, it was possible to separate the three peptides completely and demonstrate that more than 90% of Asn^{386} was deamidated in this sample. This OKT3 sample, which had been held at 37°C for 2 months, still exhibited 80% activity in the potency assay, however.

Because we were able to resolve the deamidated peptides from the undegraded peptides by mapping, we were able to measure the amount of deamidation which occurred in OKT3 subjected to various storage conditions. The two asparagine deamidations described above were found to be highly temperature dependent. Lots of OKT3 stored at 2–8°C for as long as 3 years showed 25–50% deamidation but less deamidation than material held at 30°C for 3 months. This is consistent with the temperature effect seen on the IEF pattern shifts and shifts in retention time in ion-exchange HPLC observed in OKT3 over time. The conversion of four asparagines (one in each H and L chain) in the intact antibody to aspartic acids, and smaller amounts of deamidation at other sites, could account for the observed acidic shift in the IEF bands. Since the labile asparagines are in the constant region of the antibody, their deamidation would not be expected to have a large effect on antigen binding ability. In fact, OKT3 samples which showed greater than 50% deamidation at the two major sites by peptide mapping still had acceptable activity in a potency assay. The two predominant asparagines that deamidate in OKT3 are both in Asn-Gly sequences, which are known to be especially prone to deamidation (12). The deamidation reaction proceeds through a cyclic succinimide intermediate which subsequently hydrolyzes to either the Asp or the isoAsp-containing product (8). Both products were observed in the deamidations of OKT3, the isoAsp predominating to the extent of 65–70% in both the H and the L chain deamidations. A small amount of Asn deamidation, approximately 5%, is observed even in reference standard and probably occurs during the production or purification of OKT3 (the antibody is ascites derived).

Degradation upon Storage at 2–8°C. Peptide mapping of formulated OKT3 aged under normal storage conditions (2–8°C) revealed other sites of degradation. Samples of OKT3 production lots, aged 14 months to 3 years, were analyzed by tryptic mapping. These OKT3 lots had fallen below 75% activity in the potency assay. Components in the peptide maps that significantly increased or decreased in size relative to the standard were isolated and characterized. Most of the changes on the maps that were identified were in the H chain; an example showing peptides which appear upon degradation is illustrated in Fig. 5. An additional site of Asn deamidation, H423, was found, as indicated by the appearance of a H423–441 tryptic fragment with Asp^{423} in the sequence. Several new components that appeared in the maps of aged OKT3 gave sequencing data equivalent to peptide fragments present in the digests of standard OKT3. We suspected that these peptides resulted from oxidation of labile residues, since these products often cannot be determined by conventional protein sequencing; methionine sulfoxide, for example, is reduced back to methionine by reagents used in the sequenator. Atmospheric oxygen is sufficient to oxidize Met and Cys residues in proteins. Five of the degradation peptides observed in OKT3 maps contained a methionine: L169–187, H32–38, H304–322, H347–363, and H395–411. To establish that the degradation peptide related to H32–38 was the Met^{34} sulfoxide derivative, this peptide was chemically synthesized and found to coelute with the OKT3 degradation peptide with the sequence of H32–38. Mass spectrometry was also used to confirm the

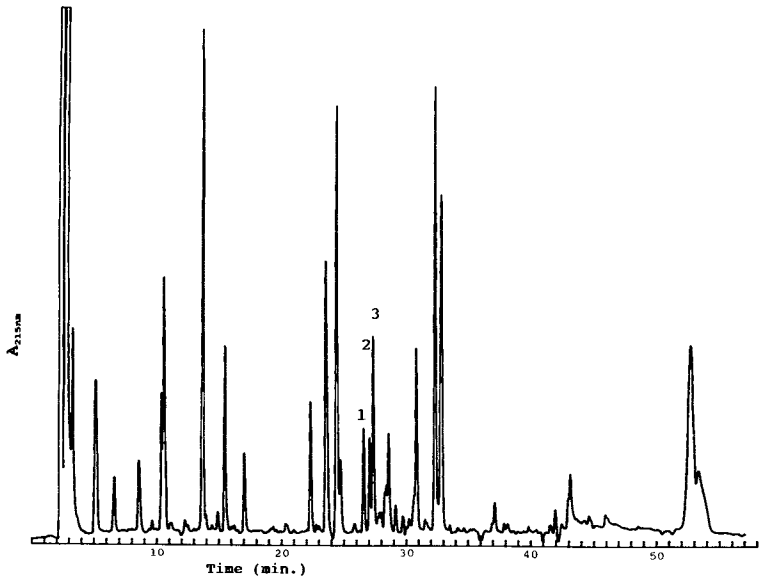

Fig. 3. OKT3 light chain map showing Asn deamidation. OKT3 heated at 37°C for 2 months was analyzed as described in the legend to Fig. 2. Peak 1 is the tryptic fragment L155–168 with Asn^{156}. Peak 2 is the Asp^{156} degradation product, and peak 3 is the $isoAsp^{156}$ product.

presence of methionine sulfoxide. The molecular weights of the degradation peptides related to H304–322, H347–363, and H395–411 were all 16 mass units higher than those calculated from the sequence data, indicating that these peptides almost certainly contained an oxidized residue, methionine sulfoxide being implicated by the facile conversion of this derivative to Met in the sequencing. The labile methionines in the peptides which appeared as degradation products are in positions H34, H316, H360, H408, and L164. One of these sites, H34, is in a complementarity determining region (CDR or hypervariable loop), so oxidation at this methionine may be expected to have an adverse effect on the biological activity of OKT3.

The most significant change in the peptide maps of OKT3 with long-term storage was a decrease in the size of the peak corresponding to H99–121 and the appearance of a new peak related to it by sequencing. This peptide does not contain Met; however, there is a non-disulfide-bonded Cys at residue 105, a likely candidate for oxidation. Due to the chemistry performed on this amino acid in preparing OKT3 for peptide mapping, the structural integrity of this Cys in the original sample could not be determined. Cys^{105} is part of the third CDR of the heavy chain, therefore, degradative structural changes at this residue may have a significant impact on the binding affinity of the antibody.

Samples of OKT3 showing large amounts of the oxidized peptides in their maps had significantly reduced activity in the antigen binding potency assay. OKT3 formulated in vials with a nitrogen overlay and stored at 5°C for 14 months showed no significant formation of the H99–121 degradation product or any of the degradation peptides attributed to methionine oxidation. Antibody stored under these conditions retained binding activity longer than materials stored without the nitrogen overlay.

Identification of Cross-Linked Peptide. Other experiments were directed toward the determination of regions of

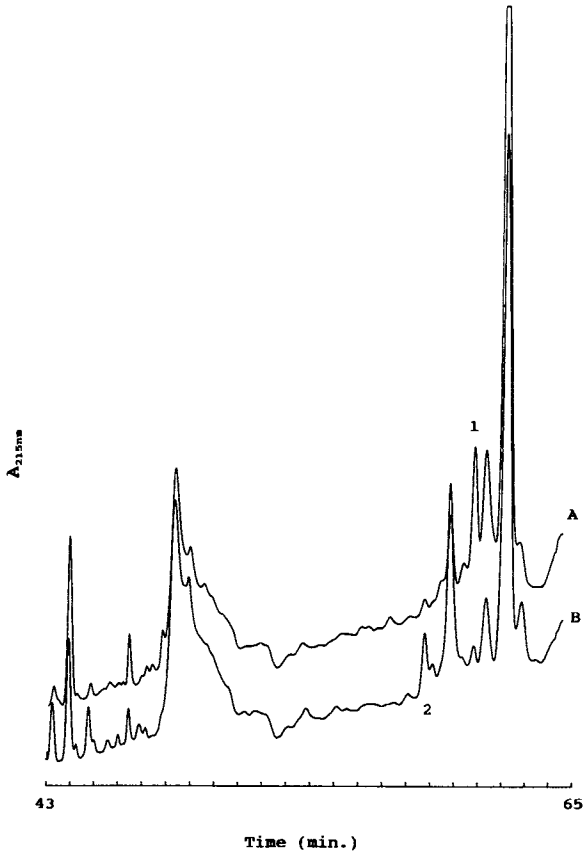

Fig. 4. OKT3 heavy chain map showing Asn deamidation. The portion of the tryptic map from 43 to 65 min is shown for clarity. Peak 1 in standard OKT3 (chromatogram A) is the H364–388 fragment which decreases in degraded OKT3 (chromatogram B). Peaks 2 are the degradation products of H364–388. The peptide mapping was done as described in the legend to Fig. 2, except the HPLC gradient was extended over 70 min.

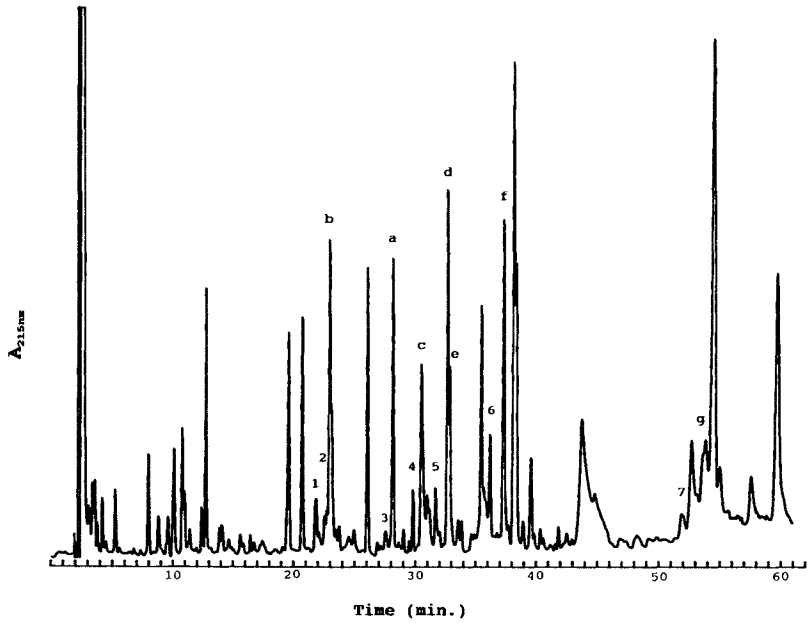

Fig. 5. Peptide map of the heavy chain of degraded OKT3. The H chain of OKT3 aged at 5°C was separated and digested with trypsin as described under Materials and Methods. For peptide mapping of the digest, the RP-HPLC column was eluted with a gradient of 5 to 45% CH_3CN in 0.1% TFA over 60 min, followed by 45 to 60% CH_3CN over 5 min. Numbered peaks are degradation peptides not present, or present in very small amounts, in the reference standard OKT3 map. They are related in sequence to the undegraded tryptic fragments (lettered) as follows: (1, a) H32–38, (2, b) H423–441, (3, c) H347–363, (4, d) H395–411, (5, e) H304–322, (6, f) H99–121, and (7, g) H364–388.

OKT3 that were involved in a degradation reaction that resulted in the formation of a cross-linked protein chain. In addition to the H and L chain bands at 52 and 26 kD that appear on SDS/PAGE of reduced OKT3, components with different molecular weight are observed in samples of degraded OKT3. These include bands with apparent molecular weights of 88, 43, 46, 17, and 6 kD. The 88-kD component is the most prominent of these degradation products and the formation of it is accelerated with increasing temperature. Appearance of this component indicates that a nonreducible covalent cross-link is formed between two chains of the antibody. A similar degradation event has been observed for other IgG antibodies (13). During the gel permeation chromatography separation of the H and L chains for peptide mapping, of OKT3 kept at 5–8°C for 3 years, a partial separation of a peak that was a shoulder on the H chain peak, corresponding to the 88-kD component, was accomplished. Several other size exclusion gels were tried but did not give a better separation of the 88-kD component. Sequencing of the 88-kD material isolated by electroblotting of an SDS/PAGE prep run gave no sequence data. This is the expected result if this component contains the amino terminus of OKT3 which has both chains blocked by pyroglutamic acid, a common occurrence in antibodies and shown to be the case in OKT3 by locating nonsequencable peptides on the L and H chain maps which had molecular weights, determined by mass spectrometry, 17 less than the expected molecular weight of the amino-terminal tryptic fragments with Gln at the amino terminus.

Peptide mapping of the material enriched in the 88-kD component showed all of the separatable peaks of both the H and the L chain tryptic fragments except for the one corresponding to L46–52. The H99–121 peak was much reduced in size, to approximately the size calculated for the amount of normal heavy chain remaining in the 88-kD sample. These results imply that the L46–52 and H99–121 regions are part of a cross-link joining the two chains. Since the cross-link appeared to be in the variable region of OKT3, we attempted to obtain a purer sample of OKT3 degradation product with the nonreducible cross-link between the H and the L chain by preparing the $F(ab')_2$ fragment of the antibody via pepsin digestion. After Cys sulfonation of purified $F(ab')_2$ fragment of degraded OKT3, gel permeation chromatography separated a component with approximately twice the molecular size of the L chain and H chain fragment, which coeluted. The size of this component indicates that it contains a nondisulfide link between two protein chains. Peptide mapping of this material compared with the map of $F(ab')_2$ of reference standard OKT3 confirmed that all of the peaks of the standard were present in the degraded material except those corresponding to L46–52 and H99–121 (Fig. 6). This result establishes that the 88-kD species is made up of equal amounts of H and L chain and that the cross-link is formed between the L46–52 and H99–121 regions. The exact chemical nature of the cross-link was not determined, but the fact that development of this degradation product is delayed under conditions that inhibit oxidation (2) suggests that an oxidative step is involved in the mechanism of its formation. Examination of known crystal structures of several antibodies reveals that portions of the regions analogous to L46–52

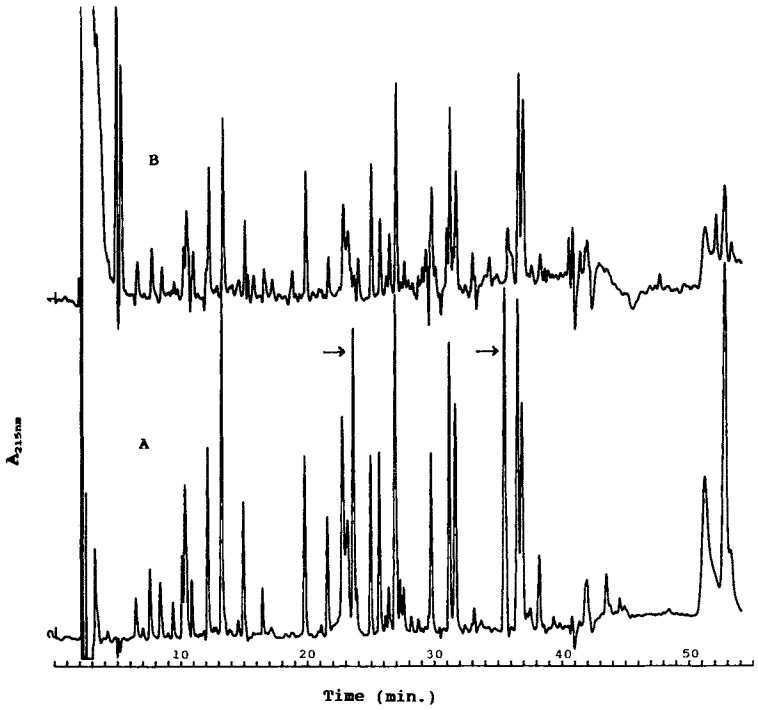

Fig. 6. Peptide maps of $F(ab')_2$ fragments. $F(ab')_2$ fragments were prepared by pepsin digestion of standard and degraded OKT3. Chromatogram A is the tryptic map of standard $F(ab')_2$. Chromatogram B is the map of the material from degraded $F(ab')_2$ with MW ~50 kD after sulfitolysis. The arrows point to peaks in the standard that are absent in the degraded sample.

and H99–121 in these antibodies are in close proximity, so it is reasonable that a cross-link could be formed as part of a degradation reaction. Since the location of the two peptides involved in the cross-link dictates that the cross-link is in or adjacent to the binding site of the antibody, it follows that formation of this cross-link could severely impair the ability of the antibody to recognize and bind to its antigen.

In summary, specific sites of chemical modification that occur upon storage of OKT3 were identified by peptide mapping and correlated with observed changes in chemical analyses and biological assays of the antibody. Under normal (2–8°C) storage conditions, oxidation appeared to be the major route of degradation of OKT3. The production of OKT3 was changed recently to incorporate vial filling with a nitrogen overlay and ongoing stability studies have demonstrated an improved stability profile of OKT3 when the material is formulated with the overlay. Currently the product has an approved stability dating of 9 months at 2–8°C. Relative to other degradation pathways of OKT3, asparagine deamidation was found to be preferentially accelerated with increasing temperature. The sensitivity of asparagine deamidation to temperature suggests that high temperature-accelerated stability studies of proteins with labile asparagines should be interpreted with caution.

Peptide mapping has proven to be a valuable tool in identifying degradation mechanisms of proteins and relating chemical changes to loss of activity. The information obtained by studying OKT3 degradation should be useful in the analysis of other antibodies through structural homology. OKT3 is an antibody of the IgG2a class, and it is expected that analogous sites of instability will be found in other murine antibodies of the same class. Antibodies of other isotypes and species also share many conserved residues; for example, residues equivalent to the Asn^{386}-Gly^{387} of OKT3 are found in mouse IgG3 and human IgG antibodies (14). Recently there have been reports of IEF acidic shifts with degradation of other murine IgG2a and IgG1 monoclonal antibodies (13) and asparagine deamidations in a CD4–human IgG hybrid molecule (15). Identification of the specific amino acid residues responsible for decomposition of a protein opens the possibility of using site-directed mutagenesis to synthesize analogues of the protein that are more stable and perhaps more commercially feasible to develop as therapeutics.

ACKNOWLEDGMENTS

We would like to thank D. Burinsky for providing FAB mass spectra analyses and J. Conboy and J. Henion of Cornell University for analyzing samples by ion spray mass spectrometry. We are grateful to C. Muccino for her technical assistance. We acknowledge P. Rao and E. Meyer for studies on OKT3 stability related to this report and many helpful discussions.

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