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Research Article

Analytical Approaches to the Study of Monoclonal Antibody Stability

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The stability of two purified monoclonal antibodies, MN12 and WT31, was investigated. The monoclonal antibodies were incubated for 32 days at different pH values (ranging from 3.0 to 10.0) at 4 and 37°C. Various analytical methods were used to assess changes in physicochemical properties of the proteins. The monoclonal antibodies were more susceptible to degradation at 37°C than at 4°C. At low pH irreversible precipitation occurred. Decomposition of the proteins was enhanced at increasing pH values in the alkaline range. This was concluded from mouse IgG-specific and antigen-specific enzyme-linked immunosorbent assays, flow cytometry, analytical gel permeation chromatography, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, isoelectric focusing, and immunoblotting. No substantial change in the apparent affinity constant of MN12 was observed, as determined by an affinity enzyme-linked immunosorbent assay. Fluorescence spectra, fluorescence polarization values, and fluorescence quenching parameters of MN12 and WT31 were not substantially affected, indicating that no major irreversible conformational changes had occurred. It was concluded that each of the techniques used has only limited value for stability assessment of monoclonal antibodies and, hence, that the application of several analytical techniques is essential to gain insight into monoclonal antibody stability.

KEY WORDS: monoclonal antibodies; stability; decomposition; characterization.

INTRODUCTION

During the last decade the number of candidate drug molecules with a peptide or protein character has been dramatically increased by the recent advances in the hybridoma and recombinant DNA technology. As a result, the role of protein chemistry is becoming increasingly important in the pharmaceutical sciences (1,2). It is being recognized by the pharmaceutical industry and regulatory authorities that the formulation of proteins for pharmaceutical applications poses a number of specific problems. One question concerns the stabilization of biologically active proteins, not only during production and purification, but also upon storage. A closely related question is: How can the stability of such products be assessed? Proteins are complicated molecules which can undergo a variety of chemical and physical degradation processes as has been reviewed recently (3). Each structural change potentially affects the biological effect, the toxicity, and the immunogenicity of a protein. To be able to monitor protein decomposition adequately, it is necessary to apply analytical techniques which are different from the conventional methods used to characterize low molecular weight drugs. Such techniques, involving biological and physicochemical tests, are required by the United States and the European Pharmacopeia for the quality control of medicinal products derived by hybridoma and recombinant DNA technology (1,4). The significance of these tests, however, has not been fully established yet.

As far as monoclonal antibodies are concerned, surprisingly little information on their stability upon storage is available up to now. Underwood and Bean (5) reported on the effect of temperature, freeze-thawing, and pH on nonpurified monoclonal antibodies in cell culture supernatants. Bruck *et al.* (6) mentioned that a certain purified monoclonal antibody was stable at 4°C over a period of 3 months. In both reports, antigen recognition was used as the only parameter to indicate the stability. Apart from some recommendations for the storage of monoclonal antibodies by Goding (7), no other data have been reported to our knowledge.

In this study, various protein characterization methods were used to determine changes in physicochemical properties of two purified monoclonal antibodies, MN12 and WT31, after storage at different pH values (ranging from 3.0 to 10.0) and temperatures (4 and 37°C). These methods in-

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clude enzyme-linked immunosorbent assay (ELISA),[5] flow cytometry, gel permeation chromatography, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing (IEF), immunoblotting, and fluorescence techniques. The major aim was to assess the potential and limitations of these techniques to indicate the stability of monoclonal antibodies.

MATERIALS AND METHODS

Chemicals

If not mentioned otherwise, chemicals used were of analytical grade.

Cell Lines, Cultivation, and Purification

Hybridoma cell line MN12H2, producing a mouse IgG_{2a} (kappa light chain) monoclonal antibody raised against the outer membrane protein P1.16 of the bacterium *Neisseria meningitidis* group B (8), was kindly provided by Dr. J. T. Poolman (Laboratory for Bacterial Vaccines, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). Hybridoma cell line WT31, producing a mouse IgG_1 (kappa light chain) monoclonal antibody which is directed against a common epitope of the human T-cell receptor (9), was a gift from Dr. W. J. M. Tax (Department of Medicine, St. Radboud Hospital, University of Nijmegen, The Netherlands). *In vitro* cultivation of the hybridoma cell lines and purification of monoclonal antibodies MN12 and WT31 to clinical grade quality have been described previously (10,11).

pH and Temperature Treatment

Solutions of 1.0 mg/ml purified MN12 or WT31 in 20 m*M* sodium phosphate pH 7.4 + 0.15 *M* sodium chloride +0.02% (w/v) sodium azide (buffer A) were adjusted to the desired pH (3.0, 4.0, 6.0, 7.4, 8.0, and 10.0) by adding 1.0 *M* hydrochloric acid or 1.0 *M* sodium hydroxide. The samples were incubated for 32 days at 4 or 37°C. After incubation the pH was readjusted to 7.4 by adding 1.0 *M* sodium hydroxide or 1.0 *M* hydrochloric acid. Next the samples were diluted with buffer A to a protein concentration of 0.5 mg/ml. After visual inspection the samples were centrifuged for 5 min at 12,000*g* to remove precipitates, if any. The clear supernatants were kept at 4°C prior to analysis. Each incubation was done in duplicate. Solutions of 1.0 mg/ml MN12 and WT31 in buffer A kept at 4°C were used as references. The supernatants and the reference solutions remained clear over the period during which the analyses were performed, indicating that no microbiological contamination had occurred.

Enzymatic Digestion

Fab and Fc fragments of MN12 and WT31 were produced by papain digestion using a modified procedure of Goding (7). Papain (2× crystallized and lyophilized powder, Sigma Chemical Corporation, St. Louis, MO) was activated by incubation of a 1.0 mg/ml solution in 0.10 *M* Tris/HCl pH 8.0 + 2.0 m*M* EDTA + 1.0 m*M* dithioerythritol (buffer B) for 20 min at 37°C. One volume of monoclonal antibody solution, whether treated or not, was dialyzed overnight at 4°C against 1000 vol of buffer B using Spectra/Por molecular porous membrane tubing, molecular weight cutoff 12,000–14,000 (Spectrum Medical Industries, Inc., Los Angeles, CA). Digestion was carried out by incubation of the dialyzed monoclonal antibody solution with the activated papain for 4 hr at 37°C using a monoclonal antibody/papain ratio of 100:1 (w/w). Digestion was terminated by addition of 0.1 vol of 0.20 *M* iodoacetamide and incubation for 1 hr on melting ice. Finally, the digested samples were extensively dialyzed against buffer A at 4°C.

Analytical Methods

Mouse IgG contents were determined by a mouse IgG-specific ELISA as described previously (10).

The antigen-binding capacity of MN12 was determined by an antigen-specific ELISA, using purified outer membrane protein P1.16 of *Neisseria meningitidis* group B (a gift from Dr. J. T. Poolman) as antigen to coat the plates. Coating was performed overnight at room temperature with 250 ng/ml P1.16 in 10 m*M* phosphate-buffered saline pH 7.3 (100 µl/well). The subsequent steps in the assay were identical to those used in the mouse IgG-specific ELISA. Antigen-binding capacities of WT31 samples were determined by flow cytometry with freshly isolated peripheral human blood lymphocytes as described before (10).

Affinity constants of MN12 for its antigen were determined by an affinity ELISA, following a procedure described by Beatty *et al.* (12). The ELISA system used was essentially similar to that for the determination of the antigen-binding capacity. Using antigen (Ag or Ag') concentrations of 250, 125, 62.5, and 31.25 ng/ml to coat the plates, maximal absorbance values proportional to the coating concentrations were obtained. Apparent affinity constants were calculated from the MN12 concentrations corresponding to 50% of maximal absorbance (OD_{50}) values of the different dilution curves according to the following equation derived by Beatty *et al.* (12):

$$K_{aff} = (n - 1)/2(n[Ab']_t - [Ab]_t) \quad (1)$$

where K_{aff} is the apparent affinity constant (M^{-1}), $n = [Ag]_t/[Ag']_t$, $[Ag]_t > [Ag']_t$, and $[Ab']_t$ and $[Ab]_t$ are the total mouse IgG concentrations at OD_{50} for antigen coating concentrations $[Ag']_t$ and $[Ag]_t$, respectively. Calculations were performed with a computer program as described by Beatty *et al.* (13).

Gel permeation chromatography was performed on a prepacked Superose HR 10/30 FPLC column (Pharmacia, Uppsala, Sweden) with spectrophotometric detection at 280 nm as described earlier (10).

SDS-PAGE was performed under reducing and nonreducing conditions with a PhastSystem and PhastGel Homogeneous 12.5 gels (Pharmacia). To determine the apparent molecular weights low molecular weight standards (Cat. No. 161-0304, Bio-Rad Laboratories, Richmond, CA) were used. IEF was performed with the same system using PhastGel IEF 3–9 gels (Pharmacia). The samples were routinely ap-

[5] Abbreviations used: ELISA, enzyme-linked immunosorbent assay; IEF, isoelectric focusing; pI, isoelectric point; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

plied at the anodic side of the gel. The pH gradient over the gels was monitored with an IEF calibration kit (Cat. No. 17-0471-01, Pharmacia). Electrophoresis and silver staining were carried out at 15°C according to the manufacturer's instructions. Contact blotting of (nonstained) protein bands to nitrocellulose membranes (BA85, 0.45 μm, Schleicher Schuell, Dassel, FRG) was accomplished by overlaying the gels with the membranes for 1 hr at 70°C (SDS-PAGE gels) or overnight at 25°C (IEF gels). Incubation of the membranes with either HRPO-labeled goat anti-mouse IgG, Fc-specific (Cat. No. 3211012, Organon Teknika, Turnhout, Belgium) or HRPO-labeled rat anti-mouse IgG, kappa light chain-specific monoclonal antibody 226 (14) (a gift from Dr. B. J. E. G. Bast, Department of Clinical Immunology, University of Utrecht, Utrecht, The Netherlands), and subsequent staining was carried out as described before (10).

Fluorescence studies were performed using an ISS GREG 200 fluorometer (ISS, Inc., Champaign, IL) equipped with a thermostated cuvet holder. All experiments were performed at 25°C. Solutions of 0.05 mg/ml monoclonal antibody in buffer A were excited at 295 nm to ensure tryptophan fluorescence only (15). The excitation slit width was 2 mm. Fluorescence emission spectra were taken from 300 to 450 nm with an emisssion slit width of 2 mm. Fluorescence maxima and relative fluorescence intensities (obtained from the area under the emission curves) were determined after subtraction of the buffer signal, using software supplied by the manufacturer of the fluorometer. A WG335 emission filter (Melles Griot, Inc., Irvine, CA) was used for fluorescence polarization and fluorescence quenching experiments. Fluorescence polarization measurements were done using an L-format configuration (16). Fluorescence polarization (P) values were computed after correction for background fluorescence and for optical or electronic differences in the parallel and perpendicular channels as described by Herron and Voss (17). Iodide quenching of tryptophan fluorescence in the range from 0 to 0.62 M potassium iodide was carried out by titration of the monoclonal antibody solutions with a freshly prepared stock solution of 2.0 M potassium iodide + 0.2 mM sodium thiosulfate. A similar titration with 2.0 M potassium chloride + 0.2 mM sodium thiosulfate was done to correct for dilution of the sample and ionic strength effects (18). Experimental quenching data were analyzed using the modified Stern-Volmer equation of Lehrer (19):

$$F_o/\Delta F = 1/f_a + 1/(f_a{}^*K_{SV}{}^*[Q]) \quad (2)$$

where F_o is the fluorescence intensity when the concentration of quencher [Q] is zero, ΔF is the difference between F_o and the fluorescence intensity in the presence of the quencher, f_a is the fraction of fluorophores accessible to the quencher, and K_{SV} is the Stern-Volmer constant. From the intercept and the slope of $F_o/\Delta F$ versus 1/[Q] plots f_a and K_{SV} were determined, respectively. Linear plots were obtained for both MN12 and WT31.

RESULTS AND DISCUSSION

When incubated at low pH, several samples of both MN12 and WT31 were turbid and contained precipitated material. This was especially true for samples of MN12 incubated at pH 3.0 (4°C), and at pH 3.0 and pH 4.0 (37°C), for the WT31 samples incubated at pH 3.0 (37°C), and for one of the duplicate WT31 samples stored at pH 3.0 (4°C) and at pH 4.0 (37°C). As the aggregates did not dissolve after readjustment of the pH to 7.4, it can be concluded that the precipitation at low pH was an irreversible process. The occurrence of precipitation corresponded with very low mouse IgG recoveries (7% or less), as shown in Fig. 1. Apart from the nonprecipitated WT31 sample incubated at pH 3.0 and 4°C (recovery, 67%), all other (nonprecipitated) samples incubated at 4°C exhibited mouse IgG recoveries around 100% (see Fig. 1A). Figure 1B shows the recoveries of the samples incubated at 37°C. According to the mouse IgG-specific ELISA, MN12 was most stable at pH 6.0, where no loss of mouse IgG content was observed. A gradual decrease in mouse IgG content with higher incubation pH values was found. WT31 turned out to be resistant to incubation at 37°C over a wider pH range (from 6.0 to 8.0) as far as the mouse IgG recovery is concerned. Only at pH 10.0 a tendency for lower recoveries was observed.

The recovery of a certain amount of mouse IgG does not necessarily mean that the detected molecules still exert their function as antibody. Therefore, the antigen-binding capacities of the MN12 and WT31 samples were determined. The results are presented in Fig. 2. As a rule, the antigen-binding recoveries of the MN12 samples showed the same trend as the mouse IgG recoveries: hardly any residual antigen-binding capacity in the precipitated samples, virtually total recoveries in the other samples incubated at 4°C, and a grad-

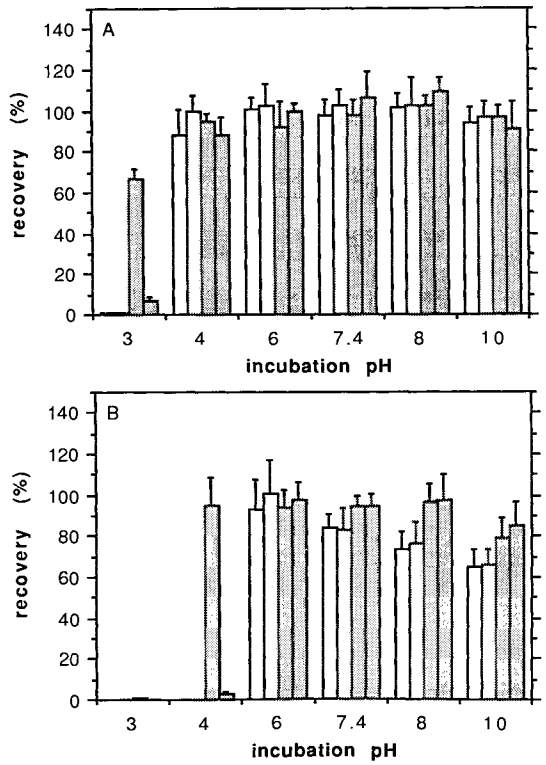

Fig. 1. Recoveries of mouse IgG of MN12 (white bars) and WT31 (shaded bars) after incubation for 32 days at different pH's at 4°C (A) and 37°C (B) as determined by ELISA. For each of the duplicate incubations the data are presented as mean + confidence interval at the level of significance $\alpha = 0.05$ of five repeated ELISA assays.

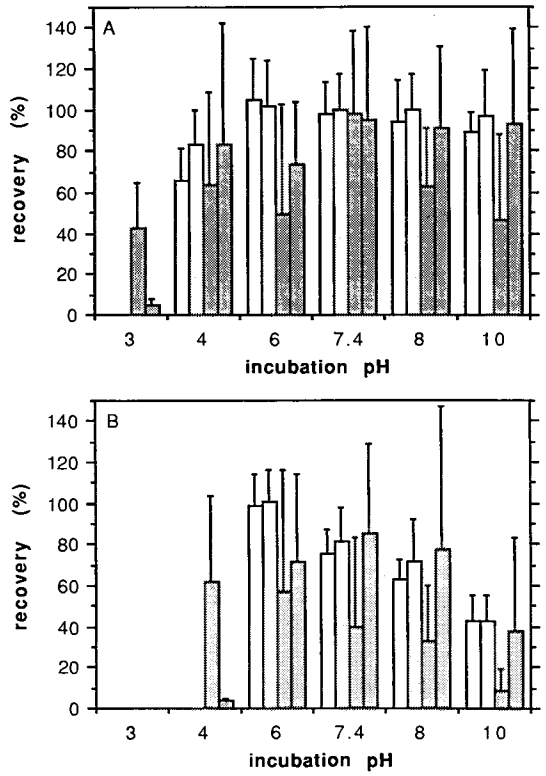

Fig. 2. Recoveries of antigen-binding capacity of MN12 (white bars) and WT31 (shaded bars) after incubation for 32 days at different pH's at 4°C (A) and 37°C (B) as determined by ELISA (MN12) and flow cytometry (WT31). For each of the duplicate incubations the data are presented as mean + confidence interval at the level of significance $\alpha = 0.05$ of three repeated ELISA assays.

ual decrease in the samples incubated at 37°C from pH 6.0 to pH 10.0. A less clear correlation between the mouse IgG content and the antigen-binding capacity was observed for WT31. Also, the data on the duplicate incubations are more diverging. This can be ascribed at least partly to a lower precision of the flow cytometric analysis when compared to the antigen-specific ELISA (cf. the heights of the confidence interval bars in Fig. 2 for MN12 and WT31).

An affinity ELISA was performed to determine the apparent affinity constants of the MN12 samples. Values for the precipitated samples could not be obtained because there was not enough reactive protein left. The log (K_{aff}) values of the nonprecipitated samples ranged from 8.2 ± 0.3 to 8.6 ± 0.2 (means ± confidence intervals at the level of significance $\alpha = 0.05$ of three repeated ELISA assays) and were not significantly different from the value of 8.5 ± 0.2 for nontreated MN12. It should be noted that the calculated apparent affinity constants may be different from the true affinity constants, because (i) the subsequent incubation and washing steps in the ELISA can shift the equilibrium and (ii) the affinity for an antigen adsorbed to a solid phase may be different from that for the same antigen in solution (20–22). Recognizing these limitations, the affinity ELISA is appropriate to determine relative differences in the affinity constants of treated and nontreated MN12 for its antigen. From the results of the mouse IgG- and the antigen-specific ELISAs it can be concluded that degradation of MN12 had occurred after incubation at 37°C at alkaline pH values. As no substantial decrease in the apparent affinity constant was observed by affinity ELISA, it was concluded that the antigen-binding site of the fraction that was recovered as mouse IgG was not substantially affected.

The presence of soluble aggregates and fragments was determined by analytical gel permeation chromatography. No fragments were detected for MN12, but some fragmentation of WT31 was observed in samples incubated at pH 7.4, 8.0, and 10.0 at 37°C (see Table I). The fragment eluted at a retention time corresponding to the retention time of the Fab fragment of WT31. It is clear from Table I that the extent of fragmentation increased with pH. The retention times of the monomeric IgG peaks of the incubated samples were not significantly different from those of the references. Small amounts of aggregates were found in the WT31 samples incubated at pH 3.0 and 4.0 at 4°C and in the MN12 samples incubated at pH 6.0 and 10.0 at 37°C. The formation of soluble aggregates might be the first step in the precipitation process that was observed in the samples incubated at low pH values.

In addition to gel permeation chromatography, SDS-PAGE was used to monitor changes in the molecular weight of the proteins. When compared to gel permeation chromatography, SDS-PAGE has a much higher resolution. On the other hand, it is performed under denaturing conditions. Boiling the samples in the presence of sodium dodecyl sulfate might induce further fragmentation or aggregation. Thus, one should be cautious in the interpretation of SDS-PAGE patterns. A difference in the band pattern from that of a reference sample can be due to either a change in the molecular weight of one or more components of the native sample or an altered sensitivity to the sample treatment. The nonprecipitated MN12 and WT31 samples incubated at 4°C showed electrophoretic patterns similar to those of the nontreated samples: a 50-kD (heavy chain) and 27-kD (light chain) band for reduced samples and a 150-kD band with a shadow band of 137 kD for nonreduced samples (see con-

Table I. Results of Analytical Gel Permeation Chromatography of MN12 and WT31 Stored for 32 Days at 4°C or 37°C at Different pH's

Storage pH	MN12		WT31	
	Aggregates[a]	Fragments[a]	Aggregates[a]	Fragments[a]
4°C				
3.0	-/-[b]	-/-	3.0/-	-/-
4.0	0/0	0/0	1.3/1.9	0/0
6.0	0/0	0/0	0/0	0/0
7.4	0/0	0/0	0/0	0/0
8.0	0/0	0/0	0/0	0/0
10.0	0/0	0/0	0/0	0/0
37°C				
3.0	-/-	-/-	-/-	-/-
4.0	-/-	-/-	0/-	-/-
6.0	0.9/0	0/0	0/0	0/0
7.4	0/0	0/0	0/0	2.5/3.3
8.0	0/0	0/0	0/0	6.0/6.8
10.0	1.5/1.7	0/0	0/0	8.8/7.4

[a] Expressed as percentage of total peak area.
[b] Not available because of a too low signal-to-noise ratio.

trols in Fig. 3). The origin of the 137-kD band is unclear but might be due to the sample treatment. In the MN12 and WT31 samples incubated at 37°C fragmentation was observed at higher incubation pH values. Moreover, the reduced samples exhibited bands with apparent molecular weights higher than that of the heavy chains. As no aggregation was found for the nonreduced samples, this suggests that intramolecular covalent bonds between the constituent chains were formed. The additional bands were more intense at increasing incubation pH values. Hence, the samples incubated at pH 10.0 (37°C) were selected to elucidate the nature of these bands by immunoblotting, using anti-Fc and anti-kappa light chain antibodies. The results are summarized in Table II. For the nonreduced samples, the 150-, 137-, and 115-kD bands were reactive with both antibodies. The 115-kD band might be an Fab/c fragment, i.e., IgG from which one Fab arm has been cleaved (23). The 39-kD and the 24-kD bands were reactive with the anti-kappa light chain antibody only and were identified as Fab fragment and kappa light chain, respectively, by comparing the relative mobilities of these bands with those of Fab fragment of MN12 (not shown). In contrast with the MN12 sample, for WT31 no Fab was detected by SDS-PAGE. This is contradictory to the results found by gel permeation chromatography, where a fragment corresponding to the molecular weight of Fab was found for WT31 and no fragmentation was observed for MN12. The 150-, 130-, 106-, and 41-kD bands of the reduced samples were not reactive with either of the two antibodies. Besides the 50-kD heavy chains (reactive with anti-Fc) and the 27-kD light chains (reactive with anti-kappa light chain), an 88-kD band reactive with both antibodies was observed for MN12 and WT31. This might be a cross-linked light and heavy chain.

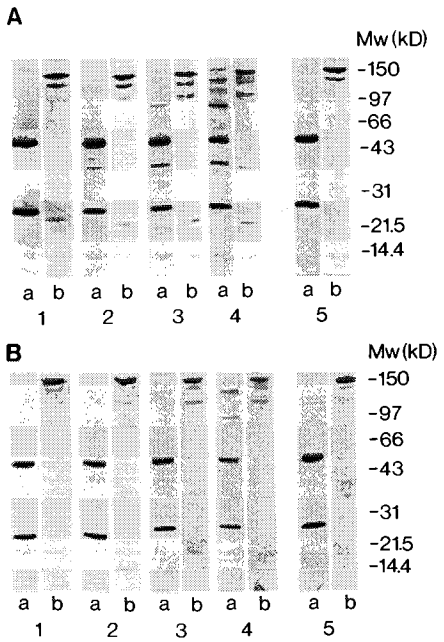

Fig. 3. Silver-stained SDS-PAGE patterns of MN12 (A) and WT31 (B) after incubation at different pH's at 37°C at pH 6.0 (lanes 1), 7.4 (lanes 2), 8.0 (lanes 3), and 10.0 (lanes 4), and of nontreated MN12 and WT31 (lanes 5). Electrophoresis was carried out under (a) reducing and (b) nonreducing conditions. The results of one of the duplicate incubations are shown.

The isoelectric points (p*I*'s) of the proteins were assessed by IEF. This electrophoretic technique, which is in contrast to SDS-PAGE performed under native conditions, has proven to be a valuable tool in the characterization of immunoglobulins (24,25). The present results are shown in Fig. 4. Both MN12 and WT31 were focused as six separate bands (see controls in Figs. 4A and B). Several reasons for this well-known p*I* heterogeneity of monoclonal antibodies have been proposed (24). The most widely accepted reason for this is carbohydrate heterogeneity, especially variation in the number of terminal sialic acid residues on the immunoglobulin heavy chain carbohydrate. The MN12 samples incubated at 4°C did not exhibit substantial p*I* changes when incubated under pH conditions ranging from 4.0 to 8.0 (see Fig. 4A). At pH 10.0 a slight acidic shift was observed. For the samples incubated at 37°C a dramatic acidic shift was observed at alkaline incubation pH values. The magnitude of this shift increased with higher incubation pH (from 6.0 to 10.0). Sample application at the cathodic instead of the anodic side on the gel did not affect the resultant patterns. This proves that the shift was not caused by precipitation during electrophoresis due to a possibly reduced solubility of the protein. A very likely reason for the appearance of a more acidic band pattern is deamidation of asparagine and glutamine residues. This involves the substitution of amide groups by carboxyl groups and, consequently, implies a decrease of the p*I* of a protein. Accelerated deamidation reactions in peptides and proteins at alkaline conditions have been well documented (3). In addition to the p*I* shift of the MN12 samples incubated at alkaline pH, the pattern became more diffuse, as shown in Fig. 5. This indicates that more subpopulations with different p*I* values were formed. The magnitude of the acidic shift was proportional to the incubation time (results not shown), which means that it concerns a continuous degradation process rather than an all-or-none phenomenon. These observations are also indicative of a deamidation mechanism, as the rate of a deamidation reaction is generally faster for asparagine than for glutamine residues and is also dependent on the local environment of the residues (3). Hence, several deamidation products will be formed in the course of time, resulting in a more diffuse IEF pattern. For WT31 a degradation pattern comparable to that for MN12 was observed (Fig. 4B). After incubation at 4°C, only the pH 10.0 sample was altered; the samples incubated at 37°C also exhibited an acidic shift at higher incubation pH's. Additionally, the p*I* shift was accompanied by an increasing number of discrete bands and by the appearance of three bands with basic p*I* values (approximately 7.9, 8.4, and 8.6). The positions of these basic bands did not depend on the incubation pH and the incubation time; the intensity increased with higher incubation pH's and with incubation time. The positions corresponded to those observed for Fab fragment of WT31 (see below).

To elucidate whether the changes in the charge of the molecules had occurred in the Fab and/or in the Fc part, IEF was performed after papain digestion of the nontreated samples and the samples incubated for 32 days at pH 10.0 at 37°C. As shown in Fig. 6, for both nontreated MN12 and WT31 a set of basic bands and a set of acidic bands were

Table II. Relative Intensities of SDS-PAGE Bands of MN12 and WT31 Incubated for 32 Days at pH 10.0 at 37°C as Detected by Silver Staining and Immunoblotting with Anti-Fc and Anti-Kappa Light Chain Antibodies[a]

		MN12			WT31		
	Apparent MW (kD)	Silver staining	Anti-Fc	Anti-kappa	Silver staining	Anti-Fc	Anti-kappa
Nonreduced samples	150	+++	+++	+++	+++	+++	+++
	137	++	++	+	+	++	+
	115	++	++	+	+	++	+
	39	+	−	++	−	−	−
	24	+	−	++	−	−	++
Reduced samples	150	+	−	−	+	−	−
	130	++	−	−	++	−	−
	106	+	−	−	+	−	−
	88	++	+	+	++	+	+
	50	+++	+++	−	+++	+++	−
	41	++	−	−	−	−	−
	27	+++	−	+++	+++	−	+++

[a] (+++) High intensity; (++) intermediate intensity; (+) weak intensity; (−) not visible.

obtained (lanes 1 and 3). Immunoblotting revealed that the basic bands were recognized by anti-kappa light chain and the acidic bands by anti-Fc antibodies. The acidic bands reacted with protein A, whereas the basic bands did not (unpublished results). Hence, the basic bands were assigned to Fab and the acidic bands to Fc fragments. The focusing of monoclonal mouse Fab and Fc as sets of basic and acidic bands, respectively, has been reported by other investigators (26,27). Whereas the heterogeneous IEF pattern of Fc fragments can be explained by the above-mentioned carbohydrate heterogeneity, the focusing of Fab as separate bands is probably due to another phenomenon. As the cleavage of immunoglobulins by papain is governed by the accessibility of the hinge region to the enzyme rather than by a specificity for certain amino acid sequences (23), the proteolytic cleavage by papain at different positions in the hinge region might be a reason for the heterogeneity in the IEF pattern of Fab fragments. The patterns of both the basic and the acidic bands of the incubated MN12 sample were changed when compared to the nontreated sample (cf. lanes 1 and 2 in Fig. 6). The acidic bands with p*I*'s ranging from 6.6 to 6.9 were shifted to more acidic positions, in the range from 6.0 to 6.6. Besides, the observed pattern was more diffuse. The four basic bands with p*I* values of 8.5 and higher were superseded by a set of three distinct bands with comparable p*I* values. Additionally, a doublet with p*I*'s of 8.25 and 8.3 and a single band with a p*I* of 7.8 were formed. For the treated WT31

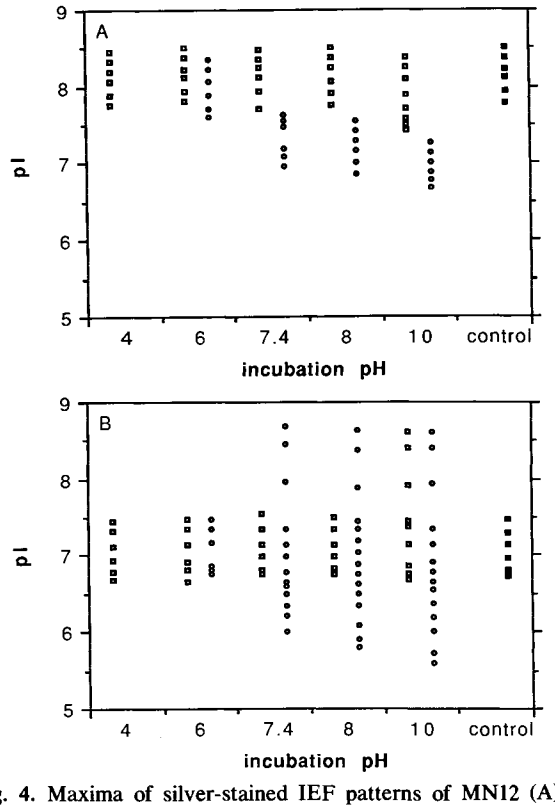

Fig. 4. Maxima of silver-stained IEF patterns of MN12 (A) and WT31 (B) after incubation for 32 days under different pH conditions at 4°C (open squares) and 37°C (open circles) and of nontreated MN12 and WT31 (filled squares). The data of one of the duplicate incubations are shown.

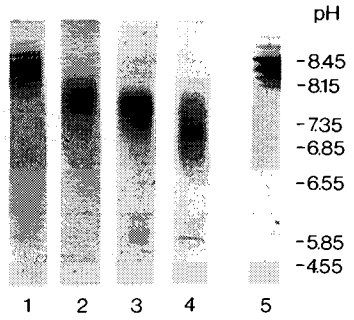

Fig. 5. Silver-stained IEF patterns of MN12 after incubation for 32 days at 37°C at pH 6.0 (lane 1), 7.4 (lane 2), 8.0 (lane 3), and 10.0 (lane 4) and of nontreated MN12 (lane 5). The results of one of the duplicate incubations are shown.

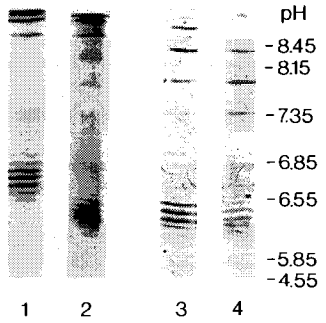

Fig. 6. Silver-stained IEF patterns of MN12 and WT31 after papain digestion. Lane 1: MN12, nontreated. Lane 2: MN12, incubated for 32 days at pH 10.0 at 37°C. Lane 3: WT31, nontreated. Lane 4: WT31, incubated for 32 days at pH 10.0 at 37°C.

sample no shift in the position of the acidic bands (p*I* range from 6.0 to 6.5) was observed, whereas the basic band pattern was altered (cf. lanes 3 and 4 in Fig. 6). In the set of the three basic bands with p*I*'s of 7.9, 8.4, and 8.6, the upper band was faded away and new band of 7.4 had appeared. Thus, the observed change in the p*I* pattern of MN12 after incubation at alkaline pH was due to molecular changes in both the Fab and the Fc part, whereas in the case of WT31 only the Fab part was substantially affected. The basic and the acidic bands of the incubated samples were still recognized by the anti-kappa light chain and the anti-Fc antibodies, respectively, as revealed by immunoblotting.

Fluorescence spectroscopy is a well-established method for investigating conformational changes in proteins (28). Here, fluorescence techniques were employed to investigate if MN12 and WT31 had undergone conformational changes after incubation. Therefore, the MN12 and WT31 samples treated at pH 4.0 (4°C) and at pH 10.0 (37°C) were chosen for comparison with nontreated MN12 and WT31. The results of the fluorescence emission spectra (emission maximum and fluorescence intensity), fluorescence polarization measurement (*P* value), and fluorescence quenching studies (f_a and K_{SV}) are listed in Table III. The emission maxima and the relative fluorescence intensities of MN12 and WT31 were not significantly influenced by pH treatment. The *P* value of MN12 was higher than that of WT31, which indicates that the tryptophan residues in the MN12 molecule have a lower overall flexibility than those in WT31. The *P* values of nontreated and treated samples, however, were not significantly different. Concerning the iodide quenching studies, comparable degrees of solvent exposure of tryptophan residues were observed for MN12 and WT31. The f_a values between 0.5 and 0.6 mean that between 50 and 60% of the total number of fluorophores present in the molecules were accessible to iodide. The quenching constant (K_{SV}) was about three times higher for MN12 than for WT31. For both MN12 and WT31, f_a and K_{SV} were not significantly affected by pH and/or temperature treatment. On the whole, the fluorescence characteristics of MN12 and WT31 were not substantially altered after incubation at high or low pH values. In comparison, the denaturation of MN12 in 6.0 *M* guanidinium hydrochloride resulted in a blue shift in the fluorescence emission maximum (to 365 nm), a sharp drop of the *P* value (to 0.13), the accessibility of iodide to all tryptophan residues (f_a = 1.0), and a dramatic increase in K_{SV} (50 M^{-1}). Thus, it can be concluded that pH treatment of MN12 and WT31 had not induced substantial irreversible changes in the conformation of the molecules.

In general, the analytical techniques used provided a consistent picture of the stability of the monoclonal antibodies. Degradation of the proteins was more pronounced at 37 than at 4°C. A maximal stability was observed at physiological or slightly acidic (pH 6.0) conditions. At lower pH values (pH 4.0 or less) irreversible precipitation occurred. When subjected to higher pH values (from 7.4 to 10.0), an enhanced degradation of the proteins was noticed by a loss of mouse IgG content and antigen-binding capacity, fragmentation, and changes in the charge of the molecules.

When examining the results in more detail, a number of comments should be made. First, some discrepancy was observed concerning the occurrence of fragmentation. Whereas IEF and gel permeation chromatography indicated the presence of Fab size structures for WT31 when subjected to alkaline pH at 37°C, this finding was not confirmed by SDS-PAGE. On the other hand, only with the latter technique were fragments observed in the corresponding MN12 samples. Furthermore, the nature of a number of bands in the SDS-PAGE patterns of these samples remains unclear. The molecular weight distribution under native conditions has been traditionally used as a major parameter to ascertain the stability of polyclonal immunoglobulin preparations (29,30). In the present study, however, analytical gel permeation chromatography turned out to be a poor "stability indicating" technique. IEF turned out to be a more valuable technique to monitor the stability of MN12 and WT31 under the conditions investigated. However, the fact that a certain

Table III. Data Obtained from Fluorescence Emission Spectra, Fluorescence Polarization Measurements, and Fluorescence Quenching Experiments of MN12 and WT31[a]

Sample	Fluorescence emission spectrum		Fluorescence polarization	Fluorescence quenching	
	Maximum (nm)	Intensity	*P* value	f_a	$K_{SV}(M^{-1})$
MN12, nontreated	353 ± 5	100 ± 10	0.25 ± 0.04	0.58 ± 0.07	10.3 ± 2.6
MN12, 32d, pH 4, 4°C	348 ± 4	97 ± 5	0.25 ± 0.02	0.57 ± 0.03	9.5 ± 2.2
MN12, 32d, pH 10, 37°C	346 ± 5	104 ± 9	0.25 ± 0.04	0.56 ± 0.05	11.0 ± 2.7
WT31, nontreated	357 ± 5	101 ± 14	0.21 ± 0.03	0.53 ± 0.14	3.3 ± 0.7
WT31, 32d, pH 4, 4°C	355 ± 7	98 ± 18	0.21 ± 0.02	0.52 ± 0.19	3.4 ± 1.4
WT31, 32d, pH 10, 37°C	357 ± 3	105 ± 12	0.22 ± 0.02	0.58 ± 0.18	3.0 ± 1.2

[a] Each value represents mean ± confidence interval at the level of significance α = 0.05 of three independent experiments.

analytical technique does not detect a change does not necessarily mean that a protein is really unchanged and still active. Also, it does not mean that such a technique is useless in the characterization of a protein. The results in this study indicate that degradation processes are monoclonal antibody dependent. Other monoclonal antibodies or other proteins may exhibit different degradation characteristics. Other storage conditions may induce different degradation processes. Thus, the application of various analytical tools is essential to be able to characterize proteins properly for quality control and the development of pharmaceutical formulations with optimum stability.

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