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*Research Article*

# Origin of the Isoelectric Heterogeneity of Monoclonal Immunoglobulin h1B4

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The origin of the microheterogeneity of a highly purified antiinflammatory humanized monoclonal antibody prepared in mammalian cell culture has been investigated. This antibody is an IgG directed toward human CD18 (a subunit of leukocyte integrins). When the IgG preparation is subjected to isoelectric focusing, it is found to contain four major species with p*I* values ranging from 6 to 7. Although the relative amounts of each form differ and some species are present only in small quantities, each has been isolated by a combination of high-resolution anion-exchange chromatography and isoelectric focusing. Comparative studies reveal no detectable differences in overall secondary (far UV circular dichroism) or tertiary (intrinsic fluorescence) structure, molecular weight (laser-desorption mass spectroscopy), or antigen binding activity. When each of the isolated species is incubated under conditions which favor deamidation, it is converted to forms of lower p*I* which appear to correspond to naturally observed species. While the isolated light chain is relatively homogeneous, the heavy chain exhibits a pattern of isoelectric focusing bands similar to that of the intact immunoglobulin. These results suggest that in this case, charge microheterogeneity is due to the sequential deamidation of the immunoglobulin heavy chain.

KEY WORDS: microheterogeneity; monoclonal antibody; deamidation; isoelectric focusing.

## INTRODUCTION

Monoclonal antibodies are under intensive development for a wide variety of therapeutic and diagnostic uses. This is due to a combination of their high intrinsic specificity, the ease of production, and recent advances in converting murine monoclonal antibodies to their humanized analogues (1–4). Although monoclonal antibodies are the product of unique genes from single clonal cell lines, it is well-known that they exhibit considerable charge heterogeneity. In this regard, it is usually found that isoelectric focusing of monoclonal antibodies resolves three to seven distinct species (5,6). The pattern of isoelectric focusing bands manifested by any individual antibody is referred to as its "spectrotype" (7).

This intrinsic heterogeneity poses a number of potential problems in the use of monoclonal antibodies as both therapeutic and diagnostic agents since the biological activity of such an antibody presumably reflects the sum of the properties of each form. It is important to investigate whether the various forms possess different antigen binding activities, effector functions, or circulation lifetimes. Although qualitative changes in spectrotype are easily detectable by analytical isoelectric focusing, it is more difficult to quantify unambiguously subtle shifts in the relative proportions of each form which might influence overall functional activity. Even though consistency of spectrotype may be sufficient for structural and functional definition of a monoclonal antibody (8), it is essential to understand the structural origin of this microheterogeneity and its potential effects upon the properties of each form.

Two major nonmutually exclusive hypotheses have been proposed to explain the presence of monoclonal antibody charge variants (9–12). According to one, microheterogeneity results from differences in glycosylation; in particular, varying contents of charged sugars such as terminal sialic acid. The second idea is that posttranslational modifications of the protein itself such as deamidation lead to forms of increasing negative charge. The spontaneous, nonenzymatic deamidation of asparagine and glutamine residues is perhaps the most commonly observed covalent change in proteins both *in vitro* and *in vivo* (13,14). The deamidation of pharmaceutical products such as human growth hormone during storage has recently been described (15). In the course of development of a humanized anti-CD18 monoclonal antibody (h1B4) as an antiinflammatory agent, studies were undertaken to characterize more fully the structural

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basis of charge heterogeneity in this protein. We report here that in the case of h1B4, the charge heterogeneity is probably due to deamidation in the immunoglobulin's heavy chain without detectable changes in protein structure or functional (antigen binding) activity.

## MATERIALS AND METHODS

*Materials.* The humanized monoclonal antibody (h1B4) was expressed in mammalian cells and purified by a combination of protein affinity chromatography and gel filtration chromatography as described previously (16,17). Murine 1B4 was propagated and purified as indicated previously (18). Iodination of murine 1B4 was carried out using chloramine-T and the protein isolated by HPLC-gel filtration chromatography. Endoglycosidase F and recombinant *N*-glycanase were obtained from Genzyme Corporation. All chemicals and reagents used were of analytical grade.

*Isoelectric Focusing.* Isoelectric focusing was performed in 1% agarose gels containing ampholytes of p*I* 5–8 (Pharmalyte) with a Pharmacia FBE-3000 flatbed apparatus thermostated at 15°C. Samples (approximately 30 μg) were focused at 15-W constant power for 1800 V-hr using a Pharmacia VH-1 volt-hour integrator. NaOH (1 *M*) was used as the catholyte, and 0.05 *M* $H_2SO_4$ as the anolyte. Gels were fixed and stained with Coomassie blue as described previously (19). Chromatograms were analyzed with a LKB Ultrascan XL laser densitometer.

*Ion-Exchange Chromatography.* Native monoclonal antibody preparation was loaded onto a Pharmacia Mono Q column (HR5/5) equilibrated with 20 m*M* Tris–HCl, pH 8.2, and eluted with a 0–0.25 *M* NaCl gradient in 20 m*M* Tris–HCl, pH 8.2. The gradient was developed over 30 min at a flow rate of 0.5 mL/min. Elution was monitored by absorbance at 280 nm and antibody-containing fractions were collected and analyzed by isoelectric focusing.

*Preparative Isoelectric Focusing.* For preparative purposes, antibody samples were loaded on a 1 × 15-cm gel strip (all sample wells); after focusing, one lane of the gel was removed and proteins were stained and identified. The corresponding unstained h1B4 containing areas were cut from the remaining gel, homogenized in the presence of phosphate-buffered saline, and extracted by shaking overnight. The insoluble agarose gel was removed, and the aqueous sample dialyzed into 10 m*M* sodium phosphate (pH 7.4) and concentrated for further investigation.

*Spectroscopic Methods.* Circular dichroic (CD) spectra were obtained with a Jasco J-720 spectropolarimeter. Each spectrum represents the average of three (baseline-corrected) scans between 184 and 260 nm using a 1-mm-path length quartz cuvette. All of the CD measurements were performed in 10 m*M* sodium phosphate, pH 7.4, at 10°C with a protein concentration of approximately 0.1 mg/mL. Fluorescence spectra were measured with either an SLM 8000 or an Hitachi F-2000 spectrofluorometer at 4- to 10-nm resolution. Solutions of immunoglobulins at 0.1 mg/mL in 10 m*M* sodium phosphate, pH 7.4, were excited at 295 nm, and emission spectra recorded at 10°C from 305 to 450 nm in a 2 × 10-mm cuvette.

*Competitive Binding Assay.* Bioactivity was assessed by competitive binding experiments conducted with phorbol myristate acetate-stimulated U-937 cells (20). Following activation, cell viability was >95% as determined by trypan blue exclusion. After washing in binding buffer (Hanks balanced salt solution containing 20 m*M* Hepes, pH 7.2, 0.14 U Aprotinin, and 2% human serum albumin), cells were incubated in $2–4 \times 10^{-11}$ *M* $^{125}$I-m1B4 in the presence of increasing concentrations of various purified species of unlabled h1B4 for 1 hr at 4°C. Cell-bound radiolabeled m1B4 was separated from unbound antibody on a sucrose gradient and quantitated using an LKB gamma counter. The $IC_{50}$ value (h1B4 concentration required to reduce binding of the labeled antibody by 50%) was calculated using a four-parameter logistic regression fit.

*Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectroscopy.* Samples were prepared by mixing 1 μL of the IgG (at 0.5–1.0 pmol/μL in 0.1% TFA) with 1 μL of matrix solution (sinapinic acid saturated in a 2:1, 0.1% TFA:ACN solution). This solution was applied to a stainless-steel insertion probe tip and allowed to air-dry. Mass spectrometric analysis was performed with a Vestec VT 2000 laser desorption time-of-flight mass spectrometer equipped with a frequency-tripled Nd:YAG laser (355 nm, 10-nsec pulse width). Spectra were obtained by signal averaging 50 consecutive laser shots (using a 200 megasample/sec transient recorder) while monitoring the shot-to-shot signal acquisition to maintain the optimal, or threshold, laser power density at the sample surface. Time to mass/charge conversion was performed using calibration curves (with horse heart apomyoglobin, MW = 16950.7, as the standard) that were generated immediately prior to acquisition of the immunoglobulin spectra. The mass assignments were taken from the centroid of the top ~30% of the ion signals and, because of the peak widths, are considered accurate to ±0.1%.

*Preparation of Carboxymethylated Heavy and Light Chains.* An antibody preparation (20–30 mg/mL) was dialyzed overnight into 0.3 *M* Tris–HCl, pH 8.2. Disulfide reduction was performed by incubating dialyzed IgG in 20 m*M* DTT for 1 hr at room temperature. Iodoacetamide was then added to a final concentration of 42 m*M* and the reaction allowed to proceed for 1 hr at 4°C in the dark. The reduced and alkylated IgG was extensively dialyzed at 4°C, first against 10 m*M* Tris, pH 8.2, and then against 10% acetic acid overnight. The dialysate was loaded onto a Pharmacia Sephacryl S-200 column and eluted with 10% acetic acid at a flow rate of 25 mL/hr. Fractions containing heavy and light chains were monitored by absorbance at 280 nm and identification was confirmed by SDS-PAGE.

*Monosaccharide Compositional Analysis.* Samples of h1B4 were hydrolyzed in 2 *N* trifluoroacetic acid (TFA) at 100°C for 15 hr. The TFA was removed from the hydrolysate by a stream of $N_2$ and samples reconstituted in deionized water. For sialic acid determination, mild hydrolysis was induced by 0.5 *N* TFA at 80°C for 70 min. Monosaccharides were analyzed using high-pH anion-exchange chromatography (HPAEC) with a Dionex BioLC unit equipped with CarboPac PA1 and guard columns as described previously (21). The detection of the separated monosaccharides was monitored by a pulsed amperometric detector using a gold electrode.

*Glycosidase Treatment.* Each isoform of h1B4 was treated with endoglycosidase F (30 mU/ml) in 0.1 *M* sodium

acetate, pH 6.0, for 1–2 days at 37°C or peptide:*N*-glycosidase F-recombinant *N*-glycanase (10 U/mL) in 0.2 *M* sodium phosphate, pH 7.2, for 16 hr at 37°C. Samples were evaluated for completeness of digestion by migration shifts employing SDS-PAGE (16% gel), compared to native untreated antibody.

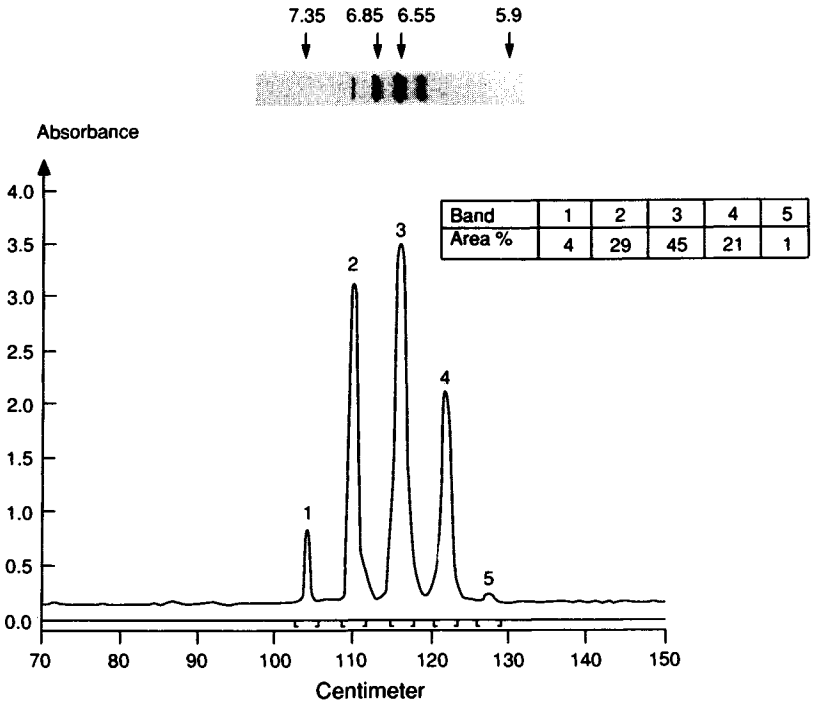
## RESULTS AND DISCUSSION

Agarose gel isoelectric focusing of h1B4 in a pH 5–8 gradient revealed five distinct isoforms of p*I* 6.2, 6.35, 6.5, 6.8, and 7.0, with most of the protein focusing in the three middle bands (Fig. 1). This spectrotype was typical and highly reproducible both qualitatively and quantitatively with h1B4 obtained from several different production batches. To characterize the individual isoforms, a high-resolution anion-exchange chromatographic method was developed to generate sufficient quantities of each isoform for further analysis. As shown in Fig. 2, when h1B4 was chromatographed on an anion-exchange column and eluted with a 0 to 0.25 *M* NaCl gradient, two major peaks (B and C), two clear shoulders (A and D), and a small, well-resolved trailing peak (E) were obtained. Isoelectric focusing of these fractions showed that each was enriched in one of the five isoforms (Fig. 2B). Further purification was performed by isoelectric focusing on individual slab gels using an entire gel for each fraction. After focusing was completed, individual bands were cut from the portion of the gel containing the major isoform in that fraction and eluted with phosphate-buffered saline. The purified isoforms were found to be homogeneous upon refocusing. No additional change in the isoelectric focusing behavior was detected for any of the isoforms during storage in 6.2 m*M* sodium phosphate, 0.15 *M* NaCl, pH 7.2, at 5°C for at least 6 months.

The overall secondary and tertiary structures of the major isoforms were examined by a combination of far-UV circular dichroism and intrinsic fluorescence emission spectra. Because of limited quantities of forms 1 and 5, CD analysis was carried out on forms 2, 3, and 4 only. The results of these studies are summarized in Table I. Forms 2, 3 and 4 display essentially the same CD spectra between 184 and 260 nm, each manifesting a minimum at 218 nm and a maximum at 202 nm with a negative shoulder near 230 nm. These characteristics are consistent with the predicted presence of extensive β-sheet. The fluorescence spectra of the four isoforms examined are also identical, displaying tryptophan emission maxima at 331 nm (Table I). Thus, by these criteria, the secondary and tertiary structures of the major h1B4 isoforms are similar if not identical.

The antigen-binding activity of the four major h1B4 isoforms was compared by a competitive binding assay (Table I). Within the rather wide experimental error of this assay (RSD of approximately 25%), all four forms bind U937 cells similarly and cannot be distinguished from the unfractionated antibody ($IC_{50}$, 1.1 n*M*).

The molecular weight of the four major forms was measured by the recently developed technique of matrix-assisted laser desorption time-of-flight mass spectroscopy (LD-TOF-MS). An average molecular weight of 150,465 was found for the four forms (see Table I). The observed variance between the four forms is less than 0.03% and well within the experimental error of the measurement (±0.1%). This corre-




| Band | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Area % | 4 | 29 | 45 | 21 | 1 |

Fig. 1. Agarose gel isoelectric focusing (pH gradient, 5–8) of native h1B4 monoclonal antibody (p*I* marker proteins are shown by arrows). A densitometric scan of the gel is shown in the lower panel and associated quantitation is summarized in the accompanying table.

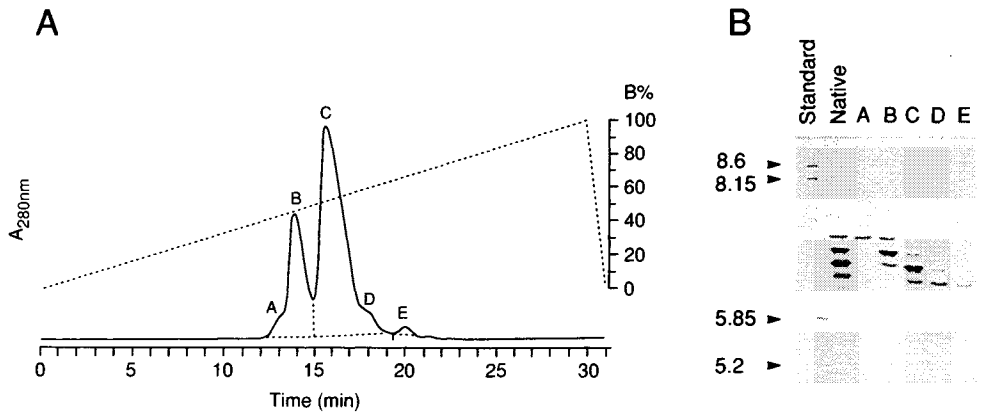



Fig. 2. (A) Chromatographic separation of h1B4 isoforms on a monoQ anion-exchange column. A gradient of 0–250 m*M* NaCl in 20 m*M* Tris-HCl (pH 8.2) was generated over 30 min at 1 mL/min. (B) Isoelectric focusing analyses of fractions A–E collected from the ion-exchange column. Reference p*I* protein markers and native h1B4 are also shown.

sponds to a maximum observed difference in molecular weight between the four forms of only 70 mass units, an amount significantly less in size than any potentially charged monosaccharide unit of triose-sized or larger. Thus, h1B4 charge heterogeneity is probably not the result of differences in carbohydrate content but, rather, result from some lower molecular weight modification of the protein.

The carbohydrate composition of the unfractioned h1B4 as well as that of the corresponding heavy and light chains was determined by high-performance anion-exchange chromatography (HPAEC). Based on previous studies, it was assumed that four *N*-acetyl glucosamine residues exist per oligosccharide chain (22). Table II shows that both the native protein and the isolated heavy chain had similar compositions. In addition, negligible quantities of sialic acid were measured. No carbohydrate could be detected in the light chain, a finding also supported by LD-TOF-MS, in which the molecular weight of the light chain was in agreement with the value calculated from the amino acid sequence (data not shown).

To eliminate any potential role for covalently attached carbohydrate in the isoelectric heterogeneity of h1B4, glycosidases were used to remove sugar residues from the protein. A representative analysis for the purified band 2 isoform is shown in Fig. 3. The native form of purified band 2 migrates as a single species upon IEF (lane B). Incubation of band 2 under deamidating conditions (pH 8 solution for 24 hr at 40°C) causes a change in a small portion of the protein, resulting in a species of lower p*I* which corresponds in migration to that of band 3 (lane C). When band 2 is incubated in the buffer used for endo F treatment for 36 hr at 37°C without enzyme addition, most of band 2 exhibits unchanged mobility (lane D). Treatment of the band 2 isoform with endoglycosidase F also has no detectable effect (lane E). In both lane D and lane E a small portion of higher p*I* material is observed. This is probably either an artifact of sample manipulation involving desalting and concentration, since this band occurs in samples subjected to desalting and concentration with or without enzyme present, or due to cyclic imide formation, which would result in the loss of a negative charge. The lower-p*I* band observed in lane E is probably due to deamidation during incubation (see below). Examination of endo F-exposed samples by SDS-PAGE shows a reduction in molecular weight consistent with carbohydrate removal. In contrast, exposure of band 2 to *N*-glycanase results in the conversion of the protein to a new species of substantially reduced p*I* (lane F). Similarly, treatment of unfractionated h1B4 with *N*-glycanase results in a similar spectrotype (i.e., same number of bands and relative intensities) but with identical acidic shifts for each of the four species (data not shown). Thus, as predicted, removal of carbohydrate by endo F which cleaves between the chitobiose units of the oligosaccharide chains does not alter the charge on the molecule. Conversely, removal of carbohydrate by *N*-glycanase leaves new acidic residues (aspartyl groups in the pep-

Table I. Biochemical and Biophysical Properties of h1B4 Isoforms

| Isoform (p*I*) | CD max/min (nm) | Fl. max (nm) | LD-TOF-MS (MW) | Binding assay $IC_{50}$ (n*M*) |
|---|---|---|---|---|
| 1 (7.0) | ND[a] | 331 | 150,450 | 1.6 |
| 2 (6.8) | 202/218 | 331 | 150,480 | 1.1 |
| 3 (6.5) | 202/218 | 331 | 150,500 | 1.2 |
| 4 (6.35) | 202/218 | 331 | 150,430 | 1.7 |

[a] Not determined.

Table II. Monosaccharide Compositional Analysis of h1B4

| Sample | Monosaccharide (mol) Fuc | GluNAc | Gal | Man | NeuAc | % total carbohydrates |
|---|---|---|---|---|---|---|
| h1B4 | 0.90 | 4 | 0.71 | 4.5 | <0.002 | 1.8 |
| h1B4, heavy chain | 0.94 | 4 | 0.7 | 4.2 | ND[a] | ND |
| h1B4, light chain | 0 | 0 | 0 | 0 | ND | |

[a] Not determined.

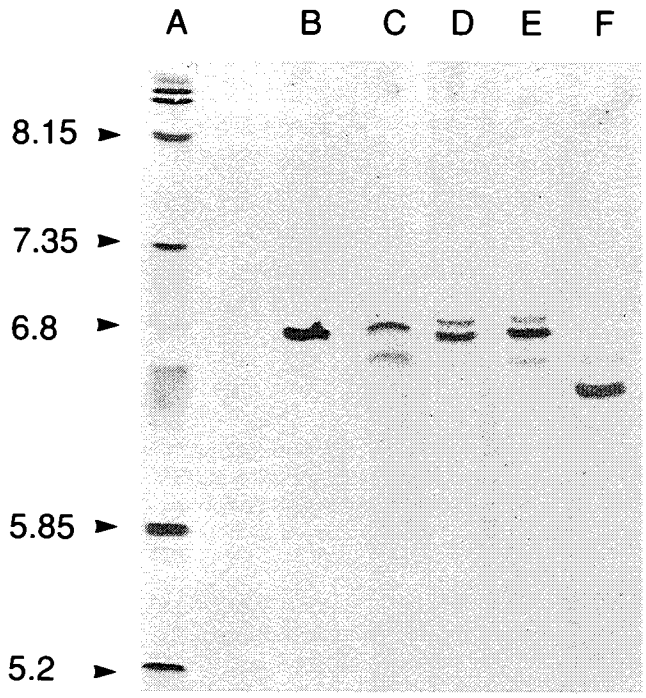



Fig. 3. Isoelectric focusing gel of purified band 2, either under deamidating conditions or after glycosidase digestion. (A) p*I* standards; (B) native band 2; (C) band 2 incubated at 40°C, pH 8, for 1 day (deamidating conditions); (D) band 2 incubated in endo F reaction buffer with no enzyme; (E) band 2 treated with endo F as in D; (F) band 2 treated with *N*-glycanase (see text for details).

tide chains), which produces the observed acidic shift. None of these results are consistent with differential glycosylation being responsible for the observed isoelectric heterogeneity of the untreated h1B4 antibody. These results do not rule out potential differences due to variable amounts of neutral sugars (23), which would not be detected as charge differences upon isoelectric focusing.

Attempts to detect deamidation in immunoglobulins directly are hampered by the very slight molecular weight change of a single dalton that results from such an event. Therefore, an indirect approach was undertaken to ascertain any potential role for deamidation in the generation of charge microheterogeneity. To this end, experiments were designed to accelerate the physiological deamidation process by incubation at higher pH and elevated temperatures. These conditions are known to favor deamidation (13–15). It was found that the individual isoforms could be converted to more acidic species by incubation at 37°C for 4 days at pH 9. As shown in Fig. 4, each isoform of h1B4 was transformed to approximately four more acidic species under these conditions. Form 1 appears to convert partially to species with p*I* values similar to forms 2, 3, and 4; form 2, to species such as 3, 4, and a lower-p*I* form; form 3, to 4 and more acidic bands; etc. These conditions lead to spectrotypes that appear related to the unfractionated native protein. Furthermore, the process is unidirectional, suggesting a sequential precursor/product relationship.

The deamidation of asparagine residues involves the formation of a cyclic imide intermediate, with the concomitant release of ammonia, followed by hydrolysis to a mixture of

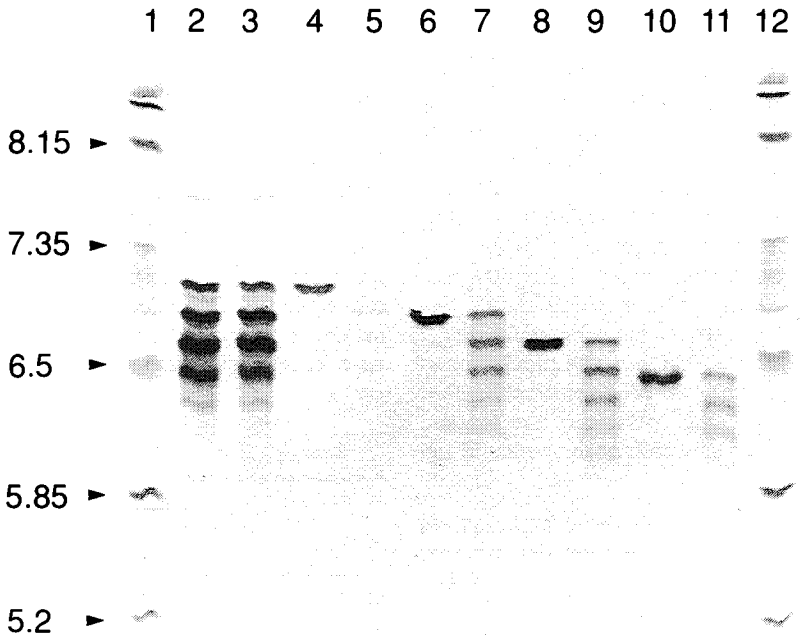



Fig. 4. Isoelectric focusing (pH 5–8 gradient) of native h1B4 and purified individual bands under normal and stress conditions. Lanes 1 and 12 are p*I* standards, lanes 2 and 3 are native h1B4, lanes 4, 6, 8, and 10 are isolated isoforms 1, 2, 3, and 4 respectively, and lanes 5, 7, 9, and 11 are the bands resulting from incubation of the individual isoforms (1–4) in 50 m*M* sodium borate, pH 9, at 37°C for 4 days.

α/β aspartyl residues (13–15). Thus, an enzymatic assay with glutamate dehydrogenase which catalyzes the formation of glutamate from 2-oxoglutarate and ammonia with concomitant oxidation of NADH can be used to measure the ammonia content of deamidated samples stored in sealed containers (24). Native h1B4 was incubated under a variety of conditions (pH 7.2 and 8 at both 30 and 40°C) for up to 12 weeks and samples were analyzed by both isoelectric focusing and ammonia content. As shown in Fig. 5, both the amount of ammonia released and the predicted ammonia evolution based on densitometry of IEF bands were measured (each new acidic band is assumed to represent native, mono, di, tri, etc., deamidated 1B4, respectively, and the theoretical ammonia evolution is taken to equal the sum of the area percentage of each band multipled by its deamidation state) (25). It can be seen that the rate of accumulation of lower-p*I* bands on IEF correlates well with the deamidation phenomena as measured by ammonia evolution.

In an attempt to localize the microheterogeneity to a subregion of the molecule, h1B4 was reduced and carboxymethylated to generate heavy and light chains. As shown in Fig. 6, the purified heavy chain focused in a series of closely spaced bands (lane C). This pattern is very similar to that seen in the unfractionated native protein (lanes B and E) but occurs in a higher-p*I* region. In contrast, the light chain is present as a single band (lane D). The heavy-chain isoforms were also isolated and found to focus as single bands. When the heavy and light chains were exposed to deamidating conditions as above, a series of lower-p*I* bands was generated by the heavy chain but the light chain remained homogeneous (not illustrated). These data suggest that a precursor/product deamidation relationship exists between the heavy-chain isoforms that is similar to that seen with the intact protein. Because the charge homogeneity of the light chain is unchanged under these conditions, it is unlikely that the light chain contributes to the spectrotype of the whole molecule. Interestingly, preliminary IEF data of $F(ab')_2$ and Fab fragments of h1B4 also show microheterogeneity but over a higher range of p*I* values. Future studies will be directed toward identification of specific deamidation sites using peptide mapping and sequencing technology.

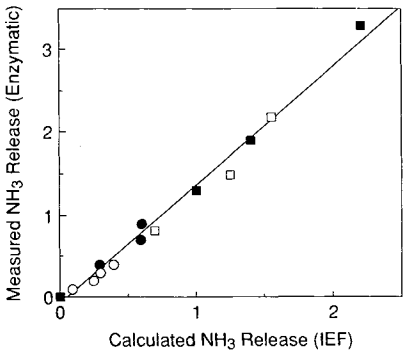



Fig. 5. Time course of ammonia release (measured vs theoretical) for native h1B4. Samples were incubated for up to 12 weeks at pH 7.2, 30°C (○); pH 7.2, 40°C (□); pH 8, 30°C (●); and pH 8, 40°C (■). The enzymatic determination of ammonia is monitored by the decrease in NADH absorbance at 340 nm (22). The theoretically predicted ammonia evolution is based upon densitometry of IEF bands (where each new acidic band is assumed to represent native, mono, di, tri, etc., deamidated h1B4, respectively). The line is drawn by linear regression and has a correlation coefficient of 0.987.

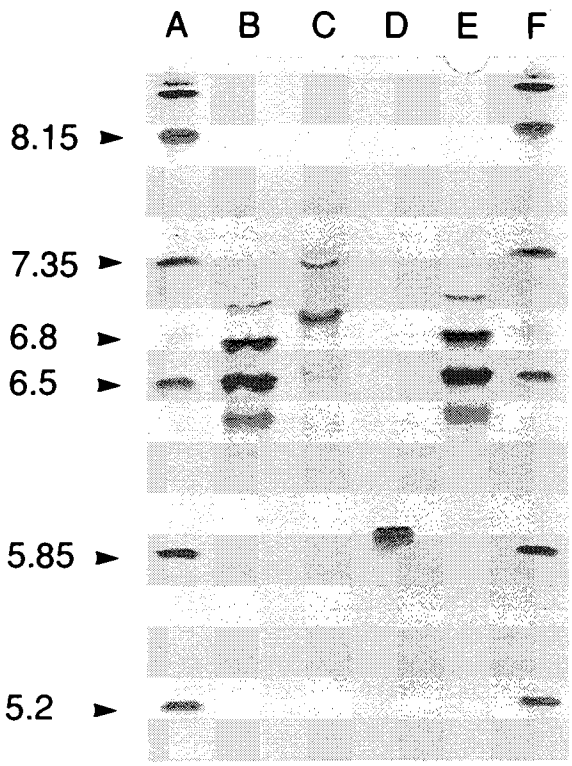



Fig. 6. Isoelectric focusing of carboxymethylated heavy and light chains. (A, F) p*I* marker; (B, E) native h1B4; (C) heavy chain; (D) light chain.

## CONCLUSIONS

Because the presence of sialic acid in h1B4 is extremely low or not detectable, it is unlikely that this sugar contributes significantly to the charge heterogeneity observed. The results of these studies strongly suggest that the charge heterogeneity of the h1B4 anti-CD18 monoclonal antibody is due primarily to deamidation of the heavy chain. These modifications are not accompanied by detectable changes in the secondary or tertiary structure or antigen binding capability of the protein. Additional properties such as effector functions (e.g., complement activation) and serum half-life remain to be assessed. It is likely that deamidation occurs spontaneously in conditioned medium during cell culture and that conditions of cell culture could greatly affect the proportion and distribution of isoelectric focusing forms (11). It is not yet known the extent to which these findings can be extended to other monoclonal immunoglobulins. Analysis of a variety of other monoclonal antibodies employing the methods used here should establish the generality or lack thereof of this explanation for the ubiquitous occurrence of the isoelectric heterogeneity of these important proteins. We caution that it is probable that in some instances differential sialation does play a role in the charge heterogeneity of certain immunoglobulins (26).

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