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Effect of Altered C_H2-associated Carbohydrate Structure on the Functional Properties and In Vivo Fate of Chimeric Mouse-Human Immunoglobulin G1

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Summary

Immunoglobulin G (IgG) molecules are glycosylated in C_H2 at Asn297; the N-linked carbohydrates attached there have been shown to contribute to antibody (Ab) stability and various effector functions. The carbohydrate attached to the IgG constant region is a complex biantennary structure. Alterations in the structure of oligosaccharide have been associated with human diseases such as rheumatoid arthritis and osteoarthritis. To study the effects of altered carbohydrate structure on Ab effector function, we have used gene transfection techniques to produce mouse-human chimeric IgG1 Abs in the Chinese hamster ovary (CHO) cell line Lec 1, which is incapable of processing the high-mannose intermediate through the terminal glycosylation steps. We also produced IgG1 Abs in Pro-5, the wild-type CHO cell line that is the parent of Lec 1. The Pro-5–produced Ab (IgG1-Pro-5) was similar to IgG1-My 1, a myeloma–produced IgG1 Ab of the same specificity, in its biologic properties such as serum half-life, ability to effect complement-mediated cytolysis, and affinity for FcγRI. Although the Lec 1–produced Ab, IgG1-Lec 1, was properly assembled and retained antigen specificity, it was incapable of complement-mediated hemolysis and was substantially deficient in complement consumption, C1q binding, and C1 activation. IgG1-Lec 1 also showed reduced but significant affinity for FcγR1 receptors. The in vivo half-life of IgG1-Lec 1 was shorter than that of either the myeloma- or Pro-5–produced counterpart, with more being cleared during the α-phase and with more rapid clearance during the β-phase. Clearance of IgG1-Lec 1 could be inhibited by the administration of yeast-derived mannan. Thus the uptake of IgG1-Lec 1 appears to be accelerated by the presence of terminally mannosylated oligosaccharide. Therefore, certain Ab functions as well as the in vivo fate of the protein are dramatically affected by altered carbohydrate structure. Expression of Igs in cell lines with defined glycosylation mutations is shown to be a useful technique for investigating the contribution of carbohydrate structure to Ab function.

All Abs are glycoproteins and are glycosylated at characteristic positions according to their isotype. The IgG molecule has one conserved glycosylation site, at Asn297, within the C_H2 domain of each of its two heavy chains. The oligosaccharides attached there are accommodated within the internal space between the two C_H2 domains (1) and are thought to stabilize the molecule and to contribute to the tertiary structure of the Fc (2). Aglycosylated IgGs have been shown to be deficient in such effector functions as complement activation, Fc receptor recognition, and Ab-dependent cell-mediated cytotoxicity (ADCC)[1] (3–5).

The oligosaccharides associated with the C_H2 of IgG are complex biantennary structures whose core structure consists of two α-mannosyl structures attached to a β-mannosyl-di-*N*-acetylchitobiose unit (see Fig. 1 *A*). The composition of the outer arms varies with the degree of terminal processing and considerable heterogeneity of carbohydrate structures is observed. This heterogeneity has been observed both in the IgG component of normal human serum (6, 7) as well as in IgG paraproteins (8). Whereas heterogeneity is always seen, the relative concentration of different carbohydrate structures is altered in some diseases. Among individuals with rheumatoid arthritis, the proportion of agalactosylated IgG sugars is increased, both in serum and synovial fluid (9, 10). Fc agalactosylation has also been observed in patients with chronic juvenile arthritis, Crohn's disease, and tuberculosis (11, 12). In some clinical studies, the degree of agalactosylation correlated with disease activity in rheumatoid arthritis patients (13). It has been proposed that the presence of agalactosylated

[1] *Abbreviations used in this paper:* ADCC, Ab-dependent cell-mediated cytotoxicity; CHO, Chinese hamster ovary; DNS, dansyl; Endo H, Endoglycosidase H; Gel-HBS, Hepes-buffered saline.

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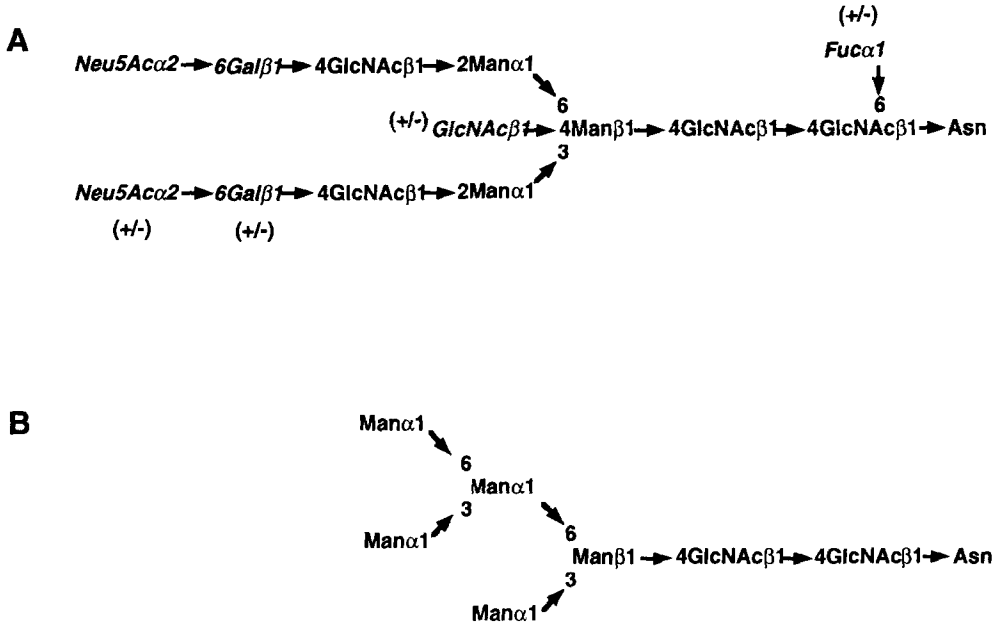

Figure 1. (*A*) Schematic representation of the complex biantennary structure characteristic of C_H2-associated oligosaccharide (7). The core residues are shown in plain type, and the terminal residues, whose variable addition confers heterogeneity upon the structure, are italicized and highlighted with (+/−) symbols. (*B*) The truncated oligosaccharide produced by the CHO glycosylation mutant Lec 1 is shown (16).

carbohydrate causes conformational changes that result in aberrant activity in the Ig, such as increased tendency for self-aggregation.

It is therefore of interest to obtain populations of Abs with defined alterations in carbohydrate structure so that the contributions of specific oligosaccharide residues to glycoprotein function can be assessed. Sequential exoglycosidase digestion of glycoprotein hormones such as choriogonadotropin has shown that successive removal of the outer sugars results in decreased biologic activity (14). Agalactosylated IgG prepared by glycosidase digestion showed decreased complement activation and Fc receptor binding activity (15). However, enzymatic or chemical treatment may damage the protein backbone. Therefore, in this study Igs were produced in Chinese hamster ovary (CHO) cells that have defined defects in oligosaccharide processing. Numerous mutants have been derived by selection for lectin resistance and mostly possess single glycosylation defects (16). These mutations occur at distinct steps in the oligosaccharide biosynthesis pathway, resulting in the accumulation of intermediate structures with reduced overall carbohydrate heterogeneity. In previous studies (17) glycosylation mutants transfected with choriogonadotropin genes produced proteins that were deficient in signal transduction.

CHO cells are widely used as recipient cells for the production of recombinant proteins. In particular, several studies have documented the production and characterization of Igs produced in CHO cells. When compared to their lymphoid cell–produced counterparts, these Abs were found to retain Ag specificity and affinity (18–20), as well as effector functions such as complement- and cell-mediated cytotoxicity (21) and Fc receptor recognition (22). Thus it was reasonable to assume that Igs produced in wild-type CHO cells are functionally equivalent to those in myeloma cells and that Ig production in the CHO glycosylation mutants would permit the study of the specific effects of altered glycosylation on otherwise normal proteins.

In the present studies chimeric mouse-human IgG1 Abs were produced in Lec 1, a CHO cell line deficient in *N*-acetylglucosaminyltransferase I activity (23). Lec 1 synthesizes oligomannosyl structures which are truncated sugars not normally found on IgG (see Fig. 1 *B*). The resulting Abs were assembled and secreted correctly, but were shown to be altered in their biologic properties and significantly deficient in numerous effector functions. Chimeric IgG1 Abs were also produced in Pro-5, the wild-type CHO parent of Lec 1. As expected, the biologic properties of these Abs were similar to their myeloma-produced counterparts.

Materials and Methods

Cell Lines. The CHO cell lines cell lines Lec 1 and Pro-5 were obtained from the American Type Culture Collection (Rockville, MD), having been deposited by Dr. Pamela Stanley (Albert Einstein College of Medicine, New York, NY) (23). The original cell lines and transfectants were maintained at 37°C under 5% CO_2 in IMDM (Irvine Scientific, Santa Ana, CA) supplemented with 5% FCS (Hyclone Laboratories, Logan, UT). The cells were maintained as monolayers on tissue culture–treated petri dishes (Falcon Labware, Lincoln Park, NJ).

The transfectoma cell line TPR1.3 (3; for clarity, referred to in this paper as IgG1-My 1, the myeloma-produced chimeric IgG1) was produced by transfecting the Ig nonproducing mouse myeloma cell line P3X63Ag8.653 with chimeric heavy and light chain genes with murine variable regions from the anti-dansyl (DNS) hybridoma 27-44 and human γ1 and κ constant regions. Cells were maintained in IMDM supplemented with 5% iron-supplemented calf serum (Hyclone).

The human monocyte-like cell line U937, which expresses FcγRI and FcγRII receptors, was maintained in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS.

Vector Design for Transfection of CHO Cells. The heavy and light chain constructs used to produce DNS-specific IgG1 chimeric Abs

in lymphoid cells were modified to allow the expression of Ig genes in nonlymphoid cells by replacing the Ig-specific promoter and enhancer with the SV40 promoter and enhancer. To construct the heavy chain vector, the 2.1-kb-BamHI fragment containing the leader and Ig promoter was subcloned into the BamHI site of pBR322. Complete digestion with EcoRI and partial digestion with HindIII removed the Ig promoter but retained the leader sequence. The SV40 promoter/enhancer was obtained from the pSV2*gpt* expression vector, where it is flanked by PvuII and HindIII restriction sites. The PvuII site at the 5′ end of the SV40 promoter was converted to an EcoRI restriction site by linker tailing. The EcoRI-HindIII fragment containing the SV40 promoter/enhancer was then cloned into the homologous restriction sites 5′ of the anti-DNS leader sequence. The BamHI-SalI fragment, which contains the V_H gene and the J-C intron, was then cloned into the BamHI and SalI sites of this pBR322 derivative, so that it adjoined the promoter/leader sequence. The heavy chain enhancer, which is flanked by two XbaI restriction sites in the J-C intron, was deleted by XbaI digestion and religation. The EcoRI-SalI fragment was then joined to human IgG1 in the expression vector pSV2ΔH*gpt* (see Fig. 2 *A*). Nucleotide sequencing of the vector showed that no mutations had been introduced in the heavy chain coding region during vector construction.

To construct the light chain vector, a PvuII site 5′ of the leader sequence but 3′ of the light chain promoter was converted to a HindIII site by linker tailing. The EcoRI-HindIII fragment encoding the SV40 promoter/enhancer was joined to the V_κ at that site. The intronic κ enhancer was deleted by EcoRI digestion and religation. The resulting light chain expression vector lacks Ig-specific controlling elements but instead contains the SV40 promoter and enhancer (see Fig. 2 *B*). Large scale plasmid purification was performed for both the expression vectors with a plasmid purification kit (Qiagen, Chatsworth, CA).

Transfection of CHO Cells by Lipofection. The Lec 1 and Pro-5 cells were each grown to 50–75% confluence on three tissue culture–treated 60 × 15-mm petri dishes. For either recipient cell line, the dishes were rinsed three times with serum-free IMDM before transfection. 30 μg each of the heavy and light chain expression vectors were combined and diluted in sterile water to a final volume of 200 μl in a polystyrene tube. 90 μl of Lipofectin (Bethesda Research Laboratories, Bethesda, MD) was then added, the contents mixed, and the tube allowed to stand at room temperature for 15 min. The mixture was then diluted to a final volume of 9 ml with serum-free IMDM. To each dish of Lec 1 or Pro-5 cells three ml of the Lipofectin mixture was added dropwise, with the drops distributed over the surface of the plate. The plates were incubated overnight at 37°C in a humidified 5% CO_2 incubator. The next day, an equal volume of IMDM with 10% FCS was added to each dish. On the following day, each plate was trypsinized and the cells diluted in 24 ml selection medium (IMDM supplemented with 10% FCS, 1% Nystatin [GIBCO BRL], 1% gentamicin and 1 mM histidinol [Sigma Chemical Co., St. Louis, MO]) and distributed 100 μl/well into 96-well flat-bottomed microtiter plates (Corning, Inc., Corning, NY). Fresh selection medium was added 3 d later. Colonies were generally seen within 10 d.

Transfectants were screened for Ab production by an ELISA using microtiter plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, VA) coated with DNS-BSA or goat anti-human IgG. The detecting Ab was alkaline phosphatase–conjugated goat anti-human κ light chain (Sigma Chemical Co.). Positive clones were expanded and subcloned.

Biosynthetic Labeling and Endoglycosidase H Hydrolysis. Secreted Abs were obtained from [^{35}S]methionine-labeled transfectants. Approximately 3 × 10^6 cells were trypsinized, washed in methionine-free DME (Irvine Scientific), and resuspended in the same medium supplemented with 15 μCi [^{35}S]methionine (Amersham, Arlington Heights, IL) and 400 μg/ml proline, and incubated at 37°C for 3 h. The supernatants were harvested and the Ab immunoprecipitated with a mixture of rabbit anti-human Fc and rabbit anti-human Fab antisera (both prepared by Letitia A. Wims in this laboratory) followed by precipitation with *Staphylococcus aureus* protein A (IgG Sorb; The Enzyme Center, Boston, MA) and washing. The Abs were resuspended in sample buffer (25 mM Tris, pH 6.7, 2% SDS, 10% glycerol, and 0.008% bromophenol blue) and eluted from protein A by boiling. The samples were analyzed by SDS-PAGE and autoradiography.

For Endoglycosidase H (Endo H) hydrolysis, ^{35}S-labeled supernatants were treated with 50 mM sodium citrate (pH 5.5), 100 mM 2-ME (Eastman Kodak Co., Rochester, NY), 2 mM PMSF (Sigma Chemical Co.), and 0.005 U Endo H (Boehringer Mannheim, Indianapolis, IN) in a final vol of 1 ml. After incubation for 24 h at 37°C, the reactions were terminated by the addition of 5

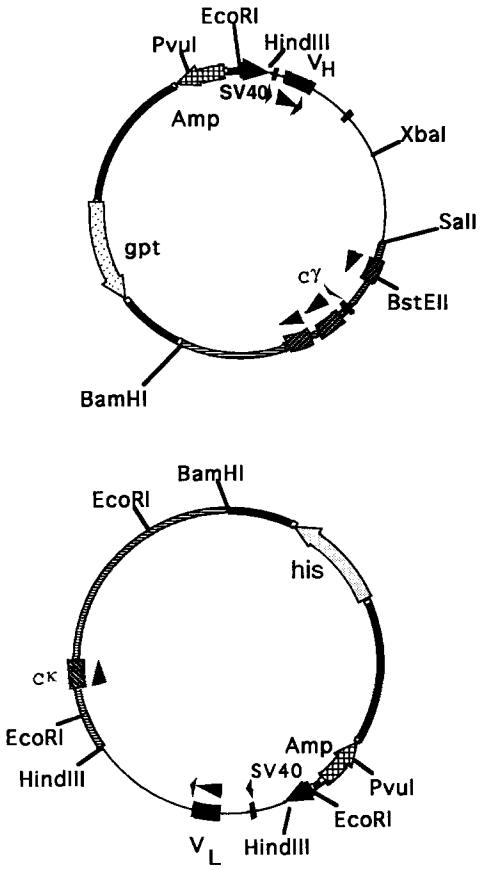

Figure 2. Vectors for the expression of Ig genes using SV40 controlling elements. The SV40 early promoter and enhancer are shown on the heavy chain (*A*) and light chain (*B*) vectors by a darkly cross-hatched arrowhead, and the murine leader and variable region genes by dark boxes. Murine intronic sequences are indicated by a thin black line. Human constant region exons are shown by diagonally striped boxes and the intervening sequences by striped black lines. The pSV2-derived sequences are represented by filled lines, with the prokaryotic selectable marker (*Amp*) and the eukaryotic selectable markers (*gpt* and *his*) shown by stippled and lightly shaded arrows, respectively. Arrows indicate the direction of transcription. Restriction sites discussed in the text are shown.

μl/sample 2 M Tris, pH 8.0. The samples were then spun briefly in a microcentrifuge to pellet debris and Ab was immunoprecipitated as described. The samples were analysed by SDS-PAGE using Tris-glycine gels.

Protein Purification. Purified Ab for biological characterization was obtained by affinity chromatography as previously described (24). Transfectoma cells were grown in roller bottle cultures in IMDM plus 2% α-calf serum (Hyclone). Supernatants from saturated cultures were buffered with 10 mM phosphate buffer (0.43 M Na_2PO_4, 0.57 M Na_2HPO_4) and supplemented with 0.45 M NaCl, 0.02 M EDTA, and 0.02% NaN_3. Supernatants were sterile filtered, degassed, and passed through AH-Sepharose columns to which the DNS isomer 2-dimethyl-aminonaphthalene-5 sulfonyl chloride had been coupled (Molecular Probes, Inc., Eugene, OR). Ab was eluted with a second DNS isomer, *N*-(5-carboxy-pentyl)-2-dimethyl-aminonaphthyl-5-sulfonamide. The bound hapten was removed by extensive dialysis against Tris-buffered saline (TBS). The concentration of purified protein was determined with the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

Measurement of Ag Affinity. A competitive binding assay was used to compare the affinity for DNS of the chimeric Abs. Immulon 2 microtiter plates were coated overnight at 4°C with DNS-BSA (5 μg/ml diluted in PBS, 50 μl/well) and blocked with 3% BSA. The murine DNS-specific Ab 27-44 was diluted to 200 ng/ml in BBS/1% BSA and mixed in equal vol with serial dilutions of IgG1-My 1, IgG1-Pro-5, or IgG1-Lec 1. The dilutions ranged from 0 to 24 μg/ml. The mixtures of murine and chimeric anti-DNSs were added in duplicate (50 μl/well) to the DNS-coated plate and incubated overnight at 4°C. Bound 27-44 Ab was detected by incubation with an alkaline phosphatase–conjugated goat anti–mouse κ Ab (Zymed Laboratories, Inc., S. San Francisco, CA), followed by the addition of substrate (*p*-nitrophenyl phosphate disodium, obtained from Sigma Chemical Co.). The data were plotted to compare the percentage of maximal binding (in the absence of competitor) to the amount of competitor present, and the point at which 50% inhibition of Ag binding by 27-44 occurred was determined. As a negative control, a chimeric dextran–specific IgG1 Ab was diluted and incubated in similar fashion with 27-44.

Measurement of Complement Fixation. The capacity of Abs for complement fixation was measured by several methods. In the direct assay, SRBC (Pocono Rabbit Farm, Canadensis, PA) were coated with DNS-BSA by the chromic chloride coupling procedure (25). The Ag-coated SRBC were loaded with ^{51}Cr, washed, and brought to a 2% suspension in Hepes-buffered saline with 0.1% gelatin (Gel-HBS). Ab at various concentrations, 0–20 μg/ml and diluted in the same buffer, was added in duplicate 50-μl aliquots to round-bottomed microtiter plates that were kept on ice throughout preparation. Chromium-loaded SRBC were added (50 μl), followed by 25 μl guinea pig complement (C′; Colorado Serum Co., Denver, CO) that had been preabsorbed with DNS-coated SRBC and adjusted to 10 LU/25 μl. Controls included spontaneous lysis (SRBC in Gel-HBS alone), total lysis (SRBC in water), SRBC incubated with Ab but without complement, and SRBC with complement and buffer alone. Experimental samples were plated in duplicate, and controls in quadruplicate. The plates were then covered and incubated in a 37°C water bath for 45 min. The plates were spun at 800 rpm in a countertop centrifuge (Beckman Instruments Inc., Fullerton, CA) to pellet red cells and debris and 50 μl from each well was withdrawn and counted in a gamma counter (model Gamma 5500; Beckman Instruments Inc.). Percent lysis was calculated as: 100 × [(mean experimental cpm − mean cpm SRBC lysis in the presence of complement alone)/(total lysis − spontaneous lysis)].

For the complement consumption assay (25) Ab (8 μg/25 μl) was incubated in duplicate with increasing amounts of DNS-BSA (0–5 μg/well) and 2 CH_{50} U of guinea pig complement in round-bottomed microtiter plates for 45 min at 37°C. [^{51}Cr]-loaded hemolysin-sensitized SRBC were then added and incubation was continued for another 45 min. The plates were centrifuged as before and the amount of ^{51}Cr release measured with a gamma counter. The percentage of complement consumption was calculated as 100 × [1−(cpm of Ab + Ag + C′/cpm Ab + C′)].

C1q Binding Assay. A solid phase assay was used to measure the capacity of Abs to bind C1q, the first step in the complement cascade (26). Immulon 2 microtiter plates were coated with 100 μl/well of DNS-BSA diluted to 10 μg/ml in PBS. The plates were then blocked with PBS plus 3% BSA overnight at 4°C and washed. The DNS-specific Abs were diluted serially from a stock concentration of 10 μg/ml in PBS plus 1% BSA, were added in duplicate 100 μl/well, and incubated overnight at 4°C. After washing with HBS, 100 μl 0.125% normal human serum diluted in HBS was added to each well and the plates were incubated at 37°C for 2 h. HBS was used for washing and as the diluent at this point because phosphate inactivates complement. Goat anti–human C1q was then diluted 1:10,000 in PBS plus 1% BSA and incubated at room temperature for 2 h, after which the plates were washed and incubated at room temperature for 2 h more with alkaline phosphatase–conjugated swine and anti-goat IgG (Boehringer Mannheim). After a final washing with PBS, substrate was added and the plates were read at 410 nm on a plate reader (model MR 700; Dynatech). IgG4, which does not bind C1q, was included on each plate as a negative control.

Fc Receptor Binding. Purified Abs (10–20 μg) were iodinated using Iodobeads (Pierce). ^{125}I was obtained from ICN (Irvine, CA). Before the assay, U937 cells were harvested, washed with PBS, and resuspended in fresh RPMI supplemented with 10% FCS and 100 U/ml recombinant human IFN-γ (the gift of Dr. Jorge Gavilondo-Cowley, Centro de Ingenieria Genetica y Biotecnologica, La Habana, Cuba). After a 48-h incubation, the cells were washed and incubated in serum-free DMEM at 37°C for 2 h to dissociate any serum Ig that might be bound to the Fc receptors. The cells were then washed again and resuspended to 5 × 10^6 cells/ml in assay buffer (Hepes buffer; 0.1 M Hepes, pH 7.4, 0.12 M NaCl, 0.005 M KCl, 0.0012 M $MgSO_4$, 0.015 M HAc, 0.01 M glucose, and 1% BSA).

The radiolabeled Ab diluted in assay buffer to ~ 10 ng/50 μl was mixed in 0.6 ml Eppendorf tubes with increasing amounts of the homologous unlabeled Ab in the same volume. Threefold dilutions from a 100-fold excess of unlabeled Ab were tested in duplicate. To measure nonspecific binding, a 1,000-fold excess of commercial human IgG (Miles Inc., Kankakee, IL) was used as inhibitor. 400 μl of stimulated U937 cells were added to each tube, and the tubes were rotated on a Labquake rotator (Labindustries, Inc., Berkeley, CA) at 18°C for 2.5 h. The tubes were then spun briefly in a microfuge, the supernatants were withdrawn, and 100 μl was measured in a gamma counter. The pellets were washed three times with PBS and the supernatant carefully removed by aspiration. Each tube was then placed in the gamma counter to measure the radioactivity retained by the pellets. Scatchard analysis was applied to determine the binding constants and number of receptors per cell (27).

In Vivo Half-Life. Female BALB/c mice were fed for at least 1 wk with water treated with potassium iodide (3 drops of 10 mg/ml per 100 ml water). For each ^{125}I-labeled protein, three mice were injected intraperitoneally with 10^6 cpm labeled Ab (28). Whole-body radioactivity was measured with a scaler/ratemeter (model

JS-5A; Wm. B. Johnson & Assoc., Ronceverte, WV) using a NaI crystal large enough to accommodate a mouse. Measurements commenced at 3 min after injection and continued at regular intervals for 300 h. The percentage of radioactive counts remaining in each sample (background subtracted) was: 100 × [(cpm from each sample)/(cpm observed immediately after injection)]. To calculate the half-life of each protein, the percentage of radioactivity remaining was plotted against the intervals at which cpm were measured.

In further experiments, mice were injected intravenously with the IgG1-Lec 1 prepared as described previously, but were coinjected with either 10 mg mannan (purified yeast polysaccharide from *Saccharomyces cerevisiae*, purchased from Sigma, and dissolved to 10 mg/100 μl PBS) or the equivalent vol of PBS (29). After 1 h, the mannan-injected mice were given a second intravenous injection of 10 mg mannan. The mice were monitored for whole-body radioactivity as before, and clearance of radioactivity over time was plotted as described.

Results

Chimeric mouse-human mAbs provide useful tools for the study of Ig function. In previous studies, the human IgG1 constant region joined to the murine DNS-specific variable region expressed in murine myeloma cells was used to investigate the functional properties of the resulting protein (30, 31). In the current study, by substituting the Ig-specific promoters and enhancers with those of SV40, the same Ab has been produced in Pro-5, a wild-type CHO cell line, and Lec 1, a variant of Pro-5 deficient in *N*-acetylglucosaminyltransferase I activity. The Ab expressed in Lec 1 will have a high-mannose carbohydrate of a type that is not normally seen on IgG attached to C_H2 in place of the normal complex carbohydrate. Thus it is possible without clinical or enzymatic modification to obtain large quantities of purified Ab with a defined, altered carbohydrate structure.

The CHO cells were refractory to electroporation, but efficient transfection was attained by lipofection. Transfectants selected for resistance to 1 mM histidinol were obtained from both Pro-5 and Lec 1. The transfectants fully assembled H_2L_2 molecules at a level ~5 μg/ml of Ab/10^6 cells/24 h, sufficient to produce adequate amounts of purified Ab from roller bottle culture. One clone of each, IgG1-Pro-5 and IgG1-Lec 1, was chosen for further study.

To confirm that carbohydrate with altered structure was present on the Lec 1-produced Ab, the Lec 1 transfectant and myeloma cells producing a chimeric Ab of the same isotype were biosynthetically labeled and the supernatants from each were treated with Endo H, which hydrolyzes high-mannose and hybrid but not complex oligosaccharides. Hydrolysis was conducted under reducing conditions to expose the sugar to the enzyme and the proteins were immunoprecipitated and analyzed by SDS-PAGE. The myeloma-produced IgG, IgG1-My 1, is mostly unaffected by treatment with Endo H although a small amount of enzyme hydrolysis is observed (Fig. 3). In contrast, the Lec 1-produced IgG, IgG1-Lec 1, is sensitive to Endo H and the heavy chain of the treated protein shows a faster mobility than the untreated one. Furthermore, the heavy chain of untreated IgG1-Lec 1 shows faster migration than does that of untreated IgG1-My 1, consistent with

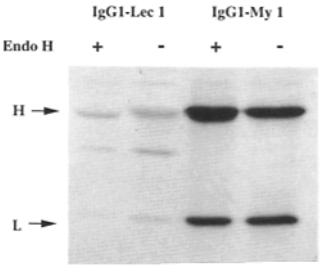

Figure 3. Endo H hydrolysis of transfectoma proteins. Transfectoma proteins were subjected to hydrolysis with Endo H under reducing conditions and were analyzed by SDS-PAGE. Heavy and light chains are indicated.

the presence of a truncated carbohydrate on IgG1-Lec 1. The identity of the band migrating between the heavy and light chains is not clear, but it appears to be a CHO cell-specific product that is not glycosylated.

To evaluate the biologic activity of these Abs in Ag-dependent assays it was necessary to confirm that the Abs' affinity for Ag is equivalent. The IgG1-Lec 1, IgG1-Pro-5, and IgG1-My 1 Abs were tested in competition experiments for their ability to inhibit Ag binding by the murine anti-DNS 27-44 with identical variable regions (Fig. 4). With all the chimeric Abs, 50% inhibition of binding by 27-44 was attained at a concentration of ~3 μg/ml. The inhibition curves of these Abs are very similar overall, thus their Ag binding capacity should be equivalent at the Ab concentrations employed in the various functional assays.

Previous work from this laboratory and others (3–5) had shown that the presence of carbohydrate in the C_H2 region of IgG1 is essential for Fc-associated functions such as complement activation and Fc receptor binding. The IgG1-Lec 1 protein containing a carbohydrate with altered structure, as well as the Pro-5–produced Ab, were now compared to the biologically active IgG1-My 1 Ab produced in murine myeloma cells.

The ability of the IgG1 Abs to activate complement was determined using several assays designed to investigate different aspects of the complement cascade. In the direct assay, which determines the ability of the Ab to complete the complement cascade and effect hemolysis, increasing amounts of Ab

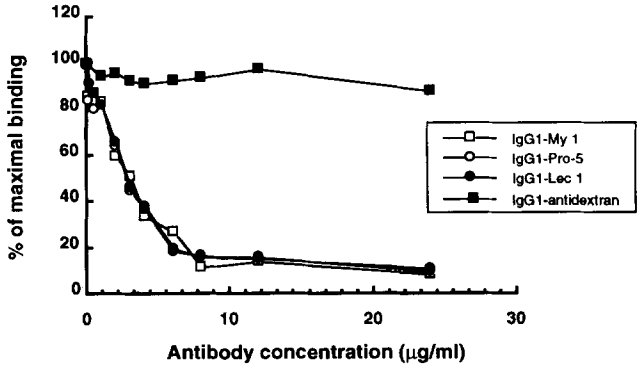

Figure 4. Competition of IgG1-My 1, IgG1-Pro-5, and IgG1-Lec 1 with the binding of murine anti-DNS 27-44 to Ag, as determined by ELISA. The data are plotted as percentage of maximum binding in the absence of competing Ab, against the increasing amount of competitor. The negative control is a chimeric IgG1 Ab specific for dextran.

were incubated with constant amounts of complement and Ag-coated SRBC. As shown in Fig. 5 *A*, red cell lysis increased as the amount of IgG1-My 1 or IgG1–Pro-5 was increased to a maximal lysis of ~35%. In contrast, IgG1-Lec 1 failed to mediate lysis at any of the concentrations tested.

In the complement comsumption assay, the capacity of Ab to deplete the complement components is measured. A constant amount of Ab is first incubated with complement in the presence of increasing amounts of Ag; if the Ag–Ab complexes consume any of the complement components, they will not be available to lyse the hemolysin-coated indicator cells. Decreased lysis therefore indicates an increase in complement activation. The ability of the IgG1-My 1, as well as IgG1–Pro-5 Ab, to consume complement is Ag dependent with total consumption ranging from of 0.5 to 50 μg Ag/well (Fig. 5 *B*). In contrast, IgG1-Lec 1 consumes only a low level of complement (<15%) in an Ag-independent manner.

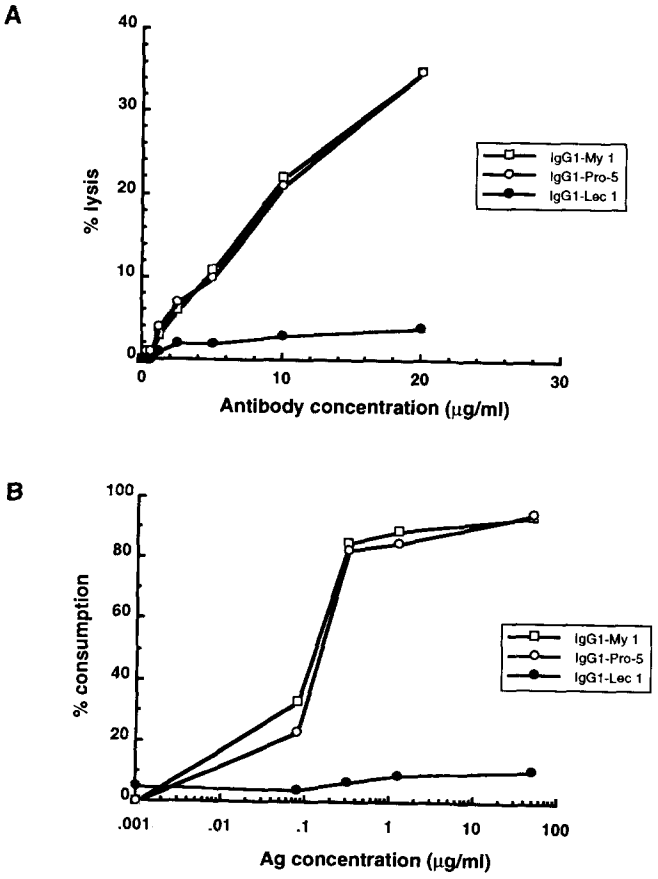

Figure 5. Complement activation by IgG1 chimeric Abs. (*A*) Direct lysis of ^{51}Cr-loaded, DNS/BSA-coated SRBC. Cells were incubated with complement and increasing amounts of Ab (in duplicate) for 45 min at 37°C. Supernatants were then harvested and the amount of lysis was quantitated with a gamma counter as described. The percent lysis is plotted against the Ab concentration. (*B*) Complement consumption. Ab (8 μg/25 μl) was incubated with complement and with increasing amounts of Ag at 37°C for 45 min. SRBC were sensitized with hemolysin, loaded with ^{51}Cr, and added to the reaction for a further incubation of 45 min. Complement consumption was calculated from chromium release as described.

To determine whether the defect in complement activation by IgG1-Lec 1 lies in the initial steps of the complement cascade, we compared the ability of these Abs to bind C1q, the first step in the activation of the classical complement pathway. Because C1q binds poorly to monomeric IgG, a solid-phase assay was used in which Ag–Ab complexes are formed on a plastic surface at sufficient density to allow C1q binding, which is then quantitated by C1q specific antiserum. An IgG4 Ab, which does not bind C1q, was included as a negative control. Both IgG1-My 1 and IgG1–Pro-5 bound C1q well, the IgG4 Ab, as expected, did not bind, and IgG1-Lec 1 showed significant but reduced C1q binding capacity (Fig. 6).

Fc Receptor Binding. To determine the affinity of binding of the IgG to the FcγRI present on IFN-γ–stimulated U937 cells, a constant amount of radiolabeled Ab was incubated with increasing amounts of unlabeled homologous ligand and the amount of radioactivity bound determined. Values for the dissociation constant and numbers of receptors per cell were obtained through Scatchard analysis; a representative experiment is shown in Fig. 7. U937 cells stimulated with IFN-γ displayed ~4.2×10^4 receptors per cell. The mean K_d of IgG1-My 1 was 1.79×10^{-9} M^{-1}, in agreement with previously published values (31). The K_d of IgG1–Pro-5 was very similar, at 1.55×10^{-9} M^{-2}. The K_d of IgG1-Lec 1 was reduced approximately four- to sixfold, with a mean value of 8.2×10^{-9} M^{-1}. Thus, the ability of the mannosylated IgG1-Lec 1 to bind FcγRI is slightly reduced but still substantial.

In Vivo Half-life. To determine the in vivo half-life of the molecules, ^{125}I-labeled Abs were introduced into BALB/c mice by intraperitoneal injection, and the amount of radioactivity present in each mouse determined by whole-body counting. The half-life values obtained by this method have been found to correlate well with those obtained by intravenous injection of labeled Abs and counting of blood samples (28). IgG1-My 1 was cleared in the β-phase with a half-life of 6.3 d (Fig. 8). In this experiment, virtually all of the IgG1-My 1 was cleared in the β-phase, but in other experiments,

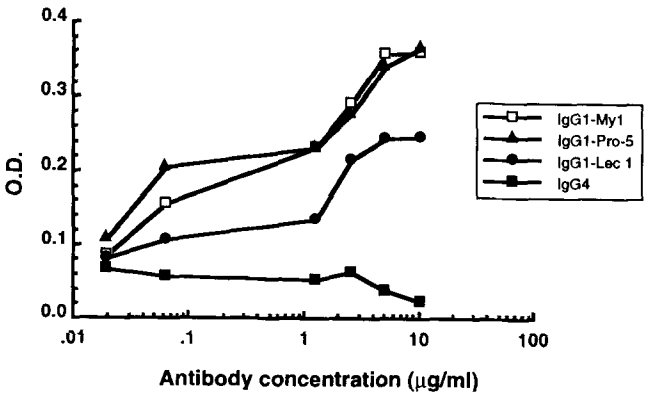

Figure 6. C1q binding by DNS-specific Abs. Binding was measured by ELISA as described using Ag-coated microtiter plates. An IgG4 Ab, which does not bind C1q, was included as a negative control.

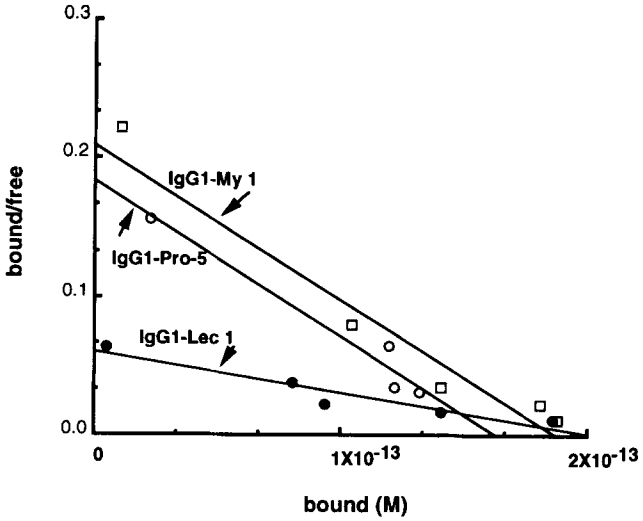

Figure 7. Scatchard analysis of the binding of IgG1-My 1 (●), IgG1-Pro-5 (○), and IgG1-Lec 1 (□) to U937 cells. The U937 cells had been stimulated for 48 h with 100 U/ml IFN-γ to increase expression of FcγR1 receptors. A constant amount of ^{125}I-labeled Ab was incubated with U937 cells with increasing amounts of unlabeled homologous competitor, and the amount of radioactivity bound to pelleted and washed cells was measured with a gamma counter. The line represents a least squares fit of the data and was calculated using Excel.

up to 40% of the protein was cleared in the α-phase. Approximately 40% of the IgG1–Pro-5 Ab was rapidly cleared in the α-phase; the remaining Ab had a β-phase half-life of 5.7 d, very similar to that of IgG1-My 1. In contrast, over 80% of IgG1-Lec 1 is consistently cleared rapidly whereas the remaining protein has a greatly reduced in vivo half-life of 4 d. Thus the IgG produced in the myeloma and Pro-5 CHO cells persists in the circulation considerably longer than does its mannosylated counterpart.

To investigate whether the rapid clearance of IgG-Lec 1

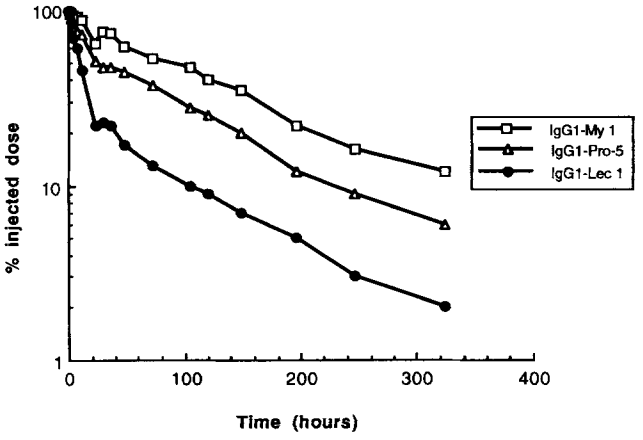

Figure 8. In vivo half-life of IgG1-My 1, IgG1–Pro-5, and IgG1-Lec 1 Abs. BALB/c mice were injected intraperitoneally with ^{125}I-labeled Ab and the persistence of radioactivity was measured by whole-body counting and is displayed as a percentage of injected dose over time. The β-phase half-life was determined from the values obtained after 48 h.

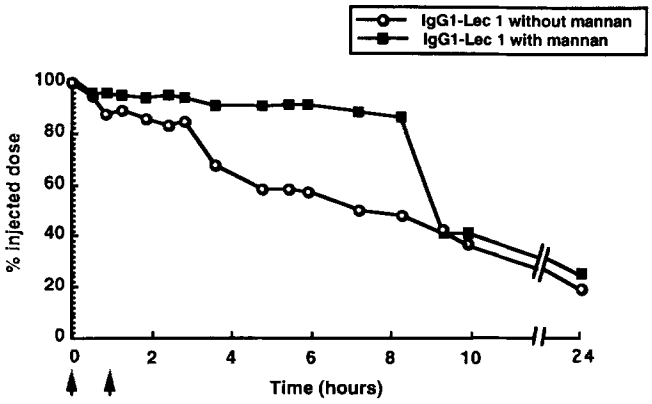

Figure 9. Effect of mannan on the in vivo clearance of IgG1-Lec 1. Mice were given intravenous injections of radiolabeled protein, either concurrently with 10 mg mannan or without. The mice receiving mannan were given a second injection 1 h later; the times of mannan injection are indicated by arrows along the *x*-axis. The percentage of radioactivity remaining was measured over time; the initial measurement was taken immediately after injection.

was mediated through mannose receptors on cells in the reticuloendothelial system, mice injected intravenously (tail vein) with ^{125}I-IgG1-Lec 1 were concurrently injected with either 10 mg of yeast mannan or with an equal volume of PBS. 1 h after injection, those mice receiving mannan were given a second injection of 10 mg mannan, and all mice were monitored by whole-body counting for 24 h. As shown in Fig. 9, the control mice injected with PBS demonstrated continuous and rapid loss of radioactivity. In contrast, the mannan-injected mice retained virtually all radioactivity for over 8 h, after which rapid clearing abruptly ensued. This result suggests that clearance takes place using the mannose receptors and these were blockaded temporarily by the yeast polysaccharide. By 24 h, the level of remaining radioactivity was the same in all the mice.

Discussion

The presence of carbohydrate in C_H2 is essential for biological activity of IgG. Although the carbohydrate attached there is heterogeneous, a considerable body of evidence shows that certain glycosylation patterns can contribute to aberrant Ig activity. Previous studies (15) had indicated that IgG rendered deficient in galactose by glycosidase treatment is deficient in C1q and Fc receptor binding. However, to rule out any diminution of Ab function that might be a consequence of the conditions of enzyme treatment, it would be desirable to produce biosynthetically Abs with defined alterations in carbohydrate structure and to determine their functional properties.

Proteins with altered carbohydrate structures can be produced in well-characterized glycosylation mutants of CHO cells (32). Wild-type CHO cells have been widely used for Ab production (18–22), so the variety of available mutants provides an expression system appropriate for the study of specific glycosylation defects of interest. Additionally, genetic engineering techniques provide the means to produce homo-

geneous populations of isotypes of interest. Therefore, we designed expression vectors for the production of DNS-specific IgG1 Abs in CHO cells. In this initial study using this experimental approach, the Igs were produced in wild-type CHO cells as well as Lec 1, a cell line incapable of processing the high mannose intermediate through the terminal glycosylation steps (Fig. 1). The resulting Abs were properly assembled and secreted and retained Ag affinity. Using this approach, Abs with novel oligosaccharide structures were made available for study.

The classical pathway of complement activation is initiated by the binding of Ab to Ag with the subsequent binding and activation of C1. Unlike the myeloma- or CHO-produced chimeric Abs, the IgG1-Lec 1 Ab was incapable of complement-mediated lysis of hapten-coated erythrocytes, although the Lec 1–produced Ab did demonstrate a reduced but significant amount of C1q binding. The C1q binding site on IgG defined by residues Glu318, Lys320, and Lys322 lies along an exposed β-strand on C_H2 (33). Other residues within this region have been shown to contribute to isotypic differences in complement activation capability (34). It is possible, therefore, that a conformational change introduced into the C_H2 domain by the altered carbohydrate serves to decrease C1q binding.

The level of C1q binding possessed by IgG1-Lec 1 might not be sufficient to activate the subsequent steps of the complement cascade. That C1q binding does not necessarily lead to cell lysis has been documented in studies of the comparative lytic efficiencies of different Ab isotypes (26, 35, 36). Additionally the IgG1-Lec 1 Ab might be an impaired substrate for interaction with complement proteins involved in the later stages of the complement cascade. The IgG1-Lec 1 differs from aglycosylated IgG1 which appears unable to bind C1q (3).

Compared to the wild-type Ab, the K_d of IgG1-Lec 1 for FcγR1 receptors on U937 cells was reduced approximately four- to sixfold. Whereas the loss of carbohydrate abolishes Fc receptor binding, changing the oligosaccharide structure has demonstrable but less drastic effects, consistent with previous reports (15, 37). Degalactosylated IgG produced by glycosidase treatment showed reduced IgG binding to U937 cells, although a binding constant was not determined (15). Intact chimeric Abs produced in yeast were as capable of ADCC as the murine counterpart, but could not activate complement (37). The oligosaccharide produced by yeast is a bulky structure with 30–100 terminal mannose residues (38) and is quite different from the truncated structure with terminal mannose residues produced by Lec 1 cells. Residues on IgG critical for Fc receptor binding lie in the lower hinge region and in the hinge proximal region, and whereas glycosylation is required for Fc receptor binding and ADCC (39–41), our results suggest that the presence of a truncated carbohydrate is sufficient to allow at least some Fc receptor binding. Additional studies are required to define precisely the carbohydrate structure necessary for different effector functions.

The in vivo fate of the IgG1-Lec 1 in mice is drastically affected by the presence of a carbohydrate of altered structure in C_H2. Whereas in vivo persistence of aglycosylated IgG1 was equivalent to that of wild type (3, 42), that of the mannosylated Ab was reduced substantially. Moreover, 80% of IgG1-Lec 1 was rapidly cleared in the α phase. Comparative in vitro protease digestion showed that IgG1-Lec 1 was no more sensitive to protease than was the wild-type Ab (data not shown). Rather, it seemed possible that clearance of IgG1-Lec 1 might occur by a different mechanism than does the clearance of IgG1-My 1 and IgG1–Pro-5.

Numerous studies have described systems for glycoprotein clearance in the liver that are localized to different compartments and that recognize different terminal sugar residues (43, 44). Liver endothelial cells bear receptors that recognize terminal mannose residues through which rapid clearance of mannosylated proteins such as ricin, OVA, and the COOH-terminal propeptide of type I procollagen has been observed (43–47). Mannose receptor–mediated clearance of glycoproteins occurred more rapidly than did clearance via galactose receptors, which were compartmentalized to liver endothelial and parenchymal cells, respectively (46, 47). Since the administration of mannan effectively inhibited the clearance of IgG1-Lec 1, although with the injection protocol used the effect was temporary, the Ab appears susceptible to uptake by mannose receptors. This result was somewhat surprising, since the Fc-associated carbohydrate normally is "buried" between the heavy chains. Indeed, the IgG1-Lec 1 Ab failed to show binding to Sepharose–Con A (data not shown). However, a report that IgG–Ag complexes injected into rats were rapidly cleared from the circulation, suggested that Ag-induced conformational changes in IgG caused the Fc carbohydrate to be exposed and susceptible to clearance by the galactose receptors on liver parenchymal cells (48). Although DNS-specific Abs are unlikely to encounter Ag in vivo, a conformational change may have been triggered in some way, perhaps through aggregation, exposing the mannose residues.

By expressing Abs in CHO cells with defined mutations in glycosylation, we have produced Abs with novel (and reasonably homogeneous) carbohydrate structures. Changing the structure of the carbohydrate may profoundly affect the behavior of Abs. In particular, the presence of a carbohydrate-bearing terminal mannose residues in C_H2 of IgG1 abolishes complement activation, attenuates Fc receptor binding, and profoundly shortens the in vivo half-life of the protein. Whereas in the current study the carbohydrate present on the IgG differed in structure from the naturally occurring carbohydrate, in future studies it will be possible to produce Abs with carbohydrate structures similar to those found on the circulating Igs in patients with certain diseases. Thus, it will be possible to determine the biologic properties of these Abs. Such studies may yield insights into the relationship between Ab structure and pathogenesis. With this technology it is possible not only to produce and study proteins with a wide variety of glycosylation defects but also to analyze a "family" of proteins, such as IgG with different isotypes, to see if effects are universal or are seen only with a particular protein. Studies like these not only may provide information about the role of carbohydrate in protein function but may also yield insights into how changes in carbohydrate structure affect protein conformation.

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