A Variant of the Dextran-Binding Mouse Plasmacytoma J558 with Altered Glycosylation of Its Heavy Chain and Decreased Reactivity with Polymeric Dextran†

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ABSTRACT: Variants have been isolated from the J558 BALB/c mouse myeloma (IgA, λ; anti-α1→3 dextran) that are producing immunoglobulin (Ig) altered in reactivity with polymeric dextran. In this report, one spontaneously arising mutant, L187, is characterized in detail. The Ig produced by L187 was indistinguishable in molecular weight from that of J558; however, a difference in charge between the two proteins was observed and could be localized to the Fab portion of the molecule. Peptide map analysis failed to show any differences between the amino acid content of the proteins; instead, the carbohydrate content of the chains differs. Examination of the glycopeptides derived from J558 and L187 Igs showed that L187 glycopeptides contain more sialic acid. Altered glycosylation apparently was the consequence of altered glycosyltransferases within the two cell lines. Comparison of the glycopeptides from the glycoprotein G of vesicular stomatitis virus (VSV) grown in mutant and wild-type cells shows that the mutant and wild-type cells differ in their cellular glycosyltransferases. Somatic cell hybrids between either L187 or J558 and MPC-11 (4T001) also demonstrate that in the proper cellular environment the J558 protein could contain large amounts of sialic acid. J558 protein, synthesized in the hybrids and containing sialic acid, bound antigen less well than did wild-type J558 protein and identically with L187 protein synthesized in the somatic hybrids. L187 Ig does not react with polymeric dextran as well as does J558 Ig. However, the mutant Ig appears to have the same reactivity pattern with oligosaccharides as does J558, and thus the size of its antibody combining site does not appear to differ from that of J558. L187 shows that different myeloma cell lines and mutants may have different glycosyltransferases available and provides the first demonstration that the carbohydrate content of an immunoglobulin molecule can influence its reactivity with antigen.

Variants producing structurally altered immunoglobulin (Ig) have been isolated from cultured myeloma cells (Cotton et al., 1973; Secher et al., 1973; Scharff et al., 1975; Preud'homme et al., 1975; Francus et al., 1978; Morrison, 1978, 1979). Most of these Igs have large alterations in chain structure, producing either deleted chains or chains with an altered constant region. Recently, the first variable-region mutant of a mouse myeloma cell line was described (Cook & Scharff, 1977). Mutants of the S107 myeloma [anti-phosphorylcholine(PC)] altered in the ability to interact with antigen were isolated, and one mutant was found to contain an amino acid substitution at the fifth residue of the J piece (M. D. Scharff, unpublished experiments).

Immunoglobulin molecules have many different binding specificities, and different antibodies can combine specifically with antigens of varying structure. The study of antibodies with specificity for dextrans has helped define the many possible sizes and the shape of the antibody combining site (Kabat, 1956; Lundblad et al., 1972). The mouse myeloma J558 produces an IgA, λ Ig, that reacts with α1→3-linked dextrans. Using cultured J558 cells, it has been possible to isolate mutants that are producing an Ig with altered reactivity with antigen. One such spontaneous mutant, L187, which produces an Ig which is altered in its glycosylation, will be described in detail in this report.

Materials and Methods

Cell Lines. The J558 tumor [IgA, λ; anti-α1→3 dextran (Lundblad et al., 1972)] was obtained from the Salk Institute, San Diego, CA, and was adapted to continuous growth in tissue culture in our laboratory. 45.6 (IgG_{2b}, κ; no known antibody activity) was established from the MPC-11 tumor by Laskov & Scharff (1970). The drug-marked derivative of 45.6, 4T001, resistant to 2.5 mM ouabain and 5 μg/mL thioguanine was obtained from M. D. Scharff, Albert Einstein College of Medicine, Bronx, NY. All subclones of J558 defective in production of Ig were isolated in this laboratory. Cells were grown in suspension culture at 37 °C in a water-saturated atmosphere of 5% CO_2 and 95% air.

Media. Cells were maintained in suspension culture in Dulbecco's modified Eagle's (DME) medium or Iscove's modified Dulbecco's (IMD) medium [Grand Island Biological Co. (GIBCO), Grand Island, NY] supplemented with 5–20% heat-inactivated (56 °C for 30 min) horse serum (HS) (GIBCO and Flow Labs, Rockville, MD), glutamine, nonessential amino acids, penicillin, and streptomycin. Rat embryo fibroblast cultures were maintained in DME medium supplemented with 10% heat-inactivated (56 °C for 30 min) fetal calf serum (FCS) (Flow Labs), penicillin, and streptomycin.

Bacterial Strains and Dextrans. Strains of *Leuconostoc mesenteroides*, that produce a mixture of dextrans B1355S and B1355L (S = 57% α1→6, 35% α1→3, and 8% α1→4; L = 88% α1→6, 3% α1→3, and 9% α1→4) or dextran B512 (95% α1→6, 5% α1→3) (Jeanes et al., 1954), were obtained from A. Jeanes, Agriculture Research Service, U.S. Department of Agriculture, Peoria, IL. The bacteria were maintained in this laboratory and used for dextran production (Jeanes, 1965). Dextrans B1355 fraction S and B512 were also obtained from A. Jeanes.

Radioactive Amino Acids and Sugars. L-[^{14}C]Leucine, L-[^{14}C]valine, L-[^{14}C]threonine, L-[^{14}C]lysine, L-[^{14}C]arginine, L-[4,5-$^{3}H_2$]leucine, L-[2,3-$^{3}H_2$]valine, and L-[^{3}H]threonine were obtained from Schwarz/Mann Radiochemicals, Becton Dickinson Immunodiagnostics, Orangeburg, NY. D-[^{14}C]-Glucosamine hydrochloride and D-[1-^{3}H]glucosamine hydro-

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chloride were obtained from Schwarz/Mann Radiochemicals or from Amersham, Arlington Heights, IL.

Enzymes. Trypsin and papain were obtained from Worthington Biochemicals, Freehold, NJ. Pronase grade B was purchased from Calbiochem, La Jolla, CA. Neuraminidase was generously supplied by S. Weitzman, State University of New York at Stony Brook, NY. Neuraminidase type IV from *Clostridium perfringens* was purchased from the Sigma Chemical Co., St. Louis, MO.

Rabbit and Sheep Antisera. Sheep anti-rabbit γ globulin (SAR) was purchased from the Pocono Rabbit Farm, Canadensis, PA. Inactivated *Staphylococcus* A (*Staph* A), used for indirect immunoprecipitation, was purchased from the Enzyme Center, Boston, MA. Antisera directed against J558 heavy (H) and light (L) chains, mouse IgA Fc fragment, and IgG_{2b} Fc fragment were raised in rabbits by repeated injections into the hind and front foot pads of 0.1 mg of purified protein in complete Freund's adjuvant. Sera were stored at −20 °C.

Detection and Isolation of Mutants. J558 cells were enriched for cells reacting poorly with dextran by removing those that formed rosettes with dextran-coated sheep red blood cells (SRBC). Dextran was oxidized by the methods of Sanderson & Wilson (1971) and coupled to SRBC by the method of Ghanta et al. (1972). Rosettes were formed as follows. J558 cells (5×10^5) were washed 3 times in DME medium without serum and resuspended in 0.2 mL of medium. An equal volume of a 10% suspension of dextran-coated SRBC was added to the myeloma cells, and the cells were pelleted by centrifugation. After 1 h on ice, nonrosetting cells were separated from rosetting cells using Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). The nonrosetting cells at the interface between the Ficoll and the medium were removed with a Pasteur pipet and rerosetted; the pelleted rosetting cells were discarded. After the fourth rosette formation, the nonrosetting cells were cloned in soft agarose by the methods of Coffino & Scharff (1971) and Coffino et al. (1971) using primary rat embryo fibroblasts as a feeder layer and medium containing 20% HS. Cloning efficiencies approaching 100% were obtained. Growing clones were overlaid with 1 mL of agarose medium containing 0.1 mL of dextrans B1355S and B1355L (2000 μg of dextran/mL). An immunoprecipitate formed around the clones that were synthesizing and secreting Ig that specifically reacted with the dextran. Clones over which no precipitate formed were recovered, grown to mass culture, and characterized.

Preparation of Labeled Immunoglobulin. Exponentially growing cells were labeled with amino acids, and the Ig was immunoprecipitated as previously described (Morrison, 1979). Alternatively, the rabbit–anti-mouse Ig-soluble complexes were isolated by adsorption for 1 h at 4 °C to inactivated *Staph* A (Kessler, 1975) followed by removal of the *Staph* A by centrifugation. The bacterial pellet was washed, suspended in 2% sodium dodecyl sulfate ($NaDodSO_4$), and boiled for 2–3 min to release labeled Ig, the bacteria were removed by centrifugation, and the supernatant was withdrawn and analyzed on $NaDodSO_4$–polyacrylamide gels.

Secreted Ig labeled with ^3H- or ^{14}C-labeled D-glucosamine hydrochloride was isolated by immunoprecipitation from the culture media of cells grown for 48 h in IMD medium supplemented with 5% HS and containing 25 μCi of [^{14}C]-glucosamine or 50 μCi of [^3H]glucosamine.

Ig without carbohydrate was isolated from the cytoplasm of cells that had been incubated for 16 h with ^{14}C- or ^3H-labeled Arg, Lys, or Val, threonine, and leucine (VTL) in the presence of a concentration of unlabeled D-glucosamine hydrochloride (10 mg/mL) that inhibits the glycosylation of nascent peptides (Klenk et al., 1972). Cells were frozen and thawed 3 times to release cell contents, and the carbohydrate minus (CHO^-) Ig was isolated by immunoprecipitation.

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gels (5 or 10%) were prepared by using a sodium phosphate buffer system, and 10% polyacrylamide gels were prepared with a Tris-HCl buffer system (Maizel, 1969). The bis(acrylamide) concentration was adjusted to 0.132% to facilitate the drying of these gels.

Tris–glycine gels without $NaDodSO_4$ were prepared as modified by Takeo (Takeo & Nakamura, 1972; Takeo & Kabat, 1978) but were cast both in a standard slab gel apparatus and in cylindrical tubes. For the preparation of affinity gels, dextran B1355 fraction S was added to the separating gel prior to polymerization.

Slab gels were dried onto Whatman 3 MM filter paper by vacuum and steam. The positions of the ^{14}C-labeled Ig peaks were determined by autoradiography and the positions of the ^3H-labeled peaks by fluorography with dimethyl sulfide (Me_2SO) and 2,5-diphenyloxazole (PPO) (Bonner & Laskey, 1974). SB-5 single-sided and XR-1 double-sided X-ray films (Eastman Kodak, Rochester, NY) were used throughout.

[^{14}C]Dextran Radioimmunoassays and Quantitation of the Amount of IgA per Cell. [^{14}C]Dextran was prepared and used in a dextran binding radioimmunoassay (RIA) (Matsuuchi & Morrison, 1978). The relative amount of cytoplasmic Ig per cell was determined by a competitive RIA using [^{14}C]-VTL-labeled J558 Ig and rabbit anti-IgA Fc and sheep anti-rabbit. Cytoplasmic lysates were made (Morrison, 1979) from J558 and L187 cells, and these lysates were used to inhibit the precipitation of ^{14}C-labeled J558.

Preparation of Dextran Affinity Columns. Affinity gels were prepared by coupling dextran B1355 to BSA–Sepharose 4B. For the preparation of these gels, 1 g of bovine serum albumin (BSA) was coupled to 100 mL of Sepharose 4B using standard procedures (Cuatrecasas et al., 1968). For the attachment of dextran to the BSA–Sepharose, 500 mg of B1355 fractions S and L (50 mg of dextran for every 10 mL of Sepharose) was dissolved in 20 mL of 0.01 M sodium acetate buffer, pH 6.0. $NaIO_4$ (50 mg) was added, and the mixture was stirred for 1 h at 25 °C. The oxidized dextran was dialyzed for 16 h at 25 °C against 0.1 M borate-buffered saline (0.1 M sodium borate and 0.15 M NaCl), pH 8.2 (BBS). BSA–Sepharose was suspended in 100 mL of BBS and mixed with the periodate-oxidized dextran for 4 h at 37 °C. $NaBH_4$ (20 mg) was added and the resin stirred for 30 min at 25 °C. The resin was washed with 2 L of distilled water and 2 L of phosphate-buffered saline (0.02 M sodium phosphate–0.15 M NaCl, pH 6.8; PBS).

Dextran Binding Assays. Dissociation experiments were performed by binding ^3H-labeled Ig from J558 and ^{14}C-labeled Ig from L187 to 20 mL of dextran–Sepharose by mixing and stirring gently in a small beaker at 25 °C for 30 mins. Nonspecifically bound material was removed by washing with PBS. Equal portions of the resin were put into small glass vials, and 200 μg of soluble dextran in PBS (B1355 S) or PBS without dextran was added. The vials were agitated repeatedly, and at various times after dextran addition, duplicate vials were removed and the immunoadsorbent was separated from the supernatant by centrifugation at 2500 rpm at 4 °C for 10 min. Bound versus free Ig was determined at each time point by counting the supernatant and the resin. Dissociation experiments were performed on whole Ig and on Fab fragments.

Precipitin Reactions. J558 or L187 cells were injected intraperitoneally into BALB/c or CDF_1 (BALB/c X DBA) (Potter et al., 1972) primed with pristane (Aldrich, Milwaukee, WI). Sera and ascitic fluid were collected and stored at −20 °C.

For precipitin reactions, the ascitic fluid from J558- or L187-injected CDF_1 mice was centrifuged exhaustively to remove insoluble material, and the fluid was stored at 4 °C with 0.01% NaN_3 added as a preservative. Precipitin and precipitin inhibition assays were performed as described by Kabat (1961); nigerose, nigerotriose, nigerotetraose, nigeropentaose, and methyl α-D-glucoside were used as inhibitors. The amount of antibody nitrogen was determined by ninhydrin (Schiffman et al., 1964).

Papain Digestion and Isolation of Fab and Fc Fragments. Mouse IgA was digested with papain essentially as described by Porter (1959). IgA Fab and Fc fragments were separated either by chromatography on DEAE-Sephadex A-25 (Pharmacia Fine Chemicals) (0.01–0.3 M $NaPO_4$, pH 8.0) or by binding to a dextran affinity column and eluting the bound Fab with 0.1 M glycine, pH 2.2.

Peptide Map Analysis. Secreted or cytoplasmic Ig labeled with [^{3}H]- or [^{14}C]VTL, -Arg, or -Lys was prepared as described previously (Morrison, 1979). Following immune precipitation, purified H and L chains and Fd or Fc fragments were isolated either on 5% $NaDodSO_4$–PO_4–polyacrylamide cylindrical gels or on Sephadex G150 columns, equilibrated in 8 M urea and 0.1 M formic acid. Digestion of the Ig and chromatography of the peptides were as previously described (Morrison & Scharff, 1975).

Preparation and Analysis of Glycopeptides. [^{3}H]- or [^{14}C]glucosamine-labeled Ig immune precipitated from the culture medium of J558 and mutant cells was suspended in 0.1 M Tris-HCl, pH 8.0. Pronase digestion was carried out at 60 °C for 48 h, as described (Weitzman et al., 1979b). For neuraminidase digestion, Pronase-digested glycopeptides were suspended in 0.2 mL of 0.05 M NH_4CO_3, pH 5.5, neuraminidase type VI from *C. perfringens* (5 μg) was added, and the mixture was incubated at 37 °C for 16 h. Glycopeptides were either chromatographed on Bio-Gel P-6 (Bio-Rad Laboratories, Richmond, CA) columns or analyzed on Tris–borate gels (Weitzman et al., 1979b). Electrophoresis was carried out for 3 h at 15 mA until the bromophenol blue marker dye was 1 cm from the bottom, the gels were dried immediately onto Whatman 3 MM filter paper, and the positions of the glycopeptide peaks were determined by autoradiography.

Vesicular Stomatitis Virus (VSV) Infection and Examination of Viral Proteins. J558 and L187 cells (10^7 cells each) were washed with IMD medium without serum. Cell pellets were incubated with 50 plaque-forming units (PFU) of VSV per cell for 30 min at 4 °C and pelleted by centrifugation, and the VSV inoculum was decanted. BSC40 (permissive monkey fibroblasts) cells were inoculated with virus by covering the monolayer with 50 PFU of VSV/cell in 0.2 mL of medium. Infected cells were incubated for 16 h with 5 mL of IMD medium supplemented with 2% dialyzed calf serum and 50 μCi of [^{14}C]glucosamine hydrochloride. After incubation, cells were removed from the medium by centrifugation and frozen and thawed twice to release virus from the cytoplasm. The viral proteins were immunoprecipitated with guinea pig anti-VSV and analyzed as indicated.

Hybrid Formation. Somatic cell hybrids between myeloma cell lines were performed by the method described by Margulies et al. (1976) and modified by Sharon et al. (1979, 1980). After hybridization, cells were incubated for 30 h in IMD medium supplemented with 20% HS, 2.5 mM ouabain (to kill L187 or J558), and hypoxanthine–aminopterin–thymidine [HAT (Littlefield, 1964), to kill the 4T001 parent]. After ouabain selection, cells were cloned in agarose in medium containing HAT. Hybrids were checked for the production of both parental Ig chains by preparing [^{14}C]VTL-labeled cytoplasmic Ig, precipitating with anti-J558 or anti-IgG_{2b} Fc and SAR, and analyzing on $NaDodSO_4$ gels.

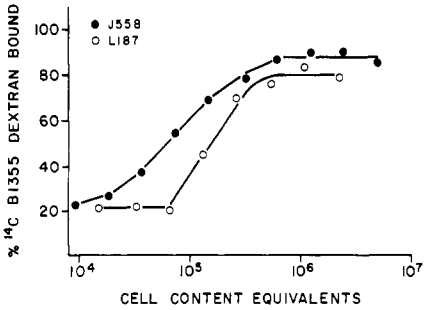

FIGURE 1: Binding of ^{14}C-labeled B1355 S and L dextran by cytoplasmic Ig from L187 and J558 cells. Varying amounts of cytoplasmic Ig were added to a constant amount of [^{14}C]dextran. Soluble immune complexes were precipitated with excess rabbit anti-mouse IgA Fc and sheep anti-rabbit IgG that was added at equivalence. The amount of radioactivity remaining in the supernatant was determined by counting in a liquid scintillation counter, and the percentage of the [^{14}C]dextran bound was calculated as described by Matsuuchi & Morrison (1978). Curves were corrected for quantitative differences in the amount of Ig per cell using a competitive RIA as described under Materials and Methods and are expressed relative to the amount of Ig per J558 cell (cell content equivalent).

Results

Isolation of Antigen Binding Mutants. J558 cells express Ig with anti-dextran activity on their membranes and will form rosettes with dextran-coated SRBC. Cells which do not form rosettes are enriched for those which no longer synthesize an Ig which binds antigen. After four cycles of separation, nonrosetting cells were cloned in soft agarose, and clones no longer secreting an Ig which reacted with dextran were identified. Of the 50 unreactive clones recovered, 48 produced only L chains, and two, L187 and L178, produced both H and L chains.

Differences in the Ability of Ig from L187 and J558 Cells to Bind Polymeric Dextran and Oligosaccharides. The mutant L187 was identified because no antigen–antibody precipitate was visible when dextran was placed in the agarose over the growing clone. Three different binding assays verified that the L187 Ig did not react with polymeric dextran as well as did J558 Ig. First, 3 times as much cytoplasmic Ig from the L187 cells as from the J558 cells was required to bind 50% of the [^{14}C]dextran used in a radioimmunoassay (Figure 1). Also, L187 Ig was found to be eluted more rapidly by soluble dextran from a solid immunoadsorbent than was J558 Ig (Figure 2A). The differences in binding did not result from different amounts of polymer in the two IgAs, since Fab produced from L187 was eluted more rapidly than Fab prepared from J558 (Figure 2B). Finally, when ^{14}C-labeled L187- and ^{3}H-labeled J558 secreted Igs were mixed, bound to dextran–Sepharose, and eluted with increasing concentrations of soluble dextran, 50% of the L187 Ig was eluted with a concentration of dextran at which more than 90% of the J558 Ig remained bound to the column (data not shown).

To determine the size of the antibody combining site of L187, we examined the ability of α1→3-linked oligosaccharides of different sizes to inhibit the precipitation of J558 or L187 Ig with polymeric dextran (Figure 3). The inhibition by nigeropentaose is greater than or equal to that by nigero-

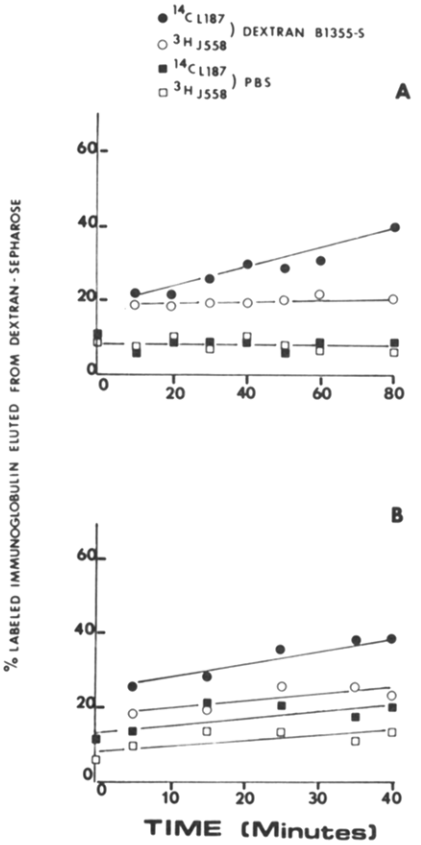

FIGURE 2: Time course of dissociation from an insoluble dextran immunoabsorbent of radiolabeled secreted Ig or Fab fragments from L187 and J558 cells. Dissociation of anti-dextran Ig from the immunoabsorbent was determined in the presence of either unlabeled dextran or phosphate-buffered saline (PBS) as described. (A) Intact Ig; (B) Fab fragments. Fab fragments were prepared following papain cleavage as described under Materials and Methods. (●) ^{14}C-Labeled L187 Ig eluted with dextran; (○) ^{3}H-labeled J558 Ig eluted with dextran; (■) ^{14}C-labeled L187 Ig eluted with PBS; (□) ^{3}H-labeled J558 Ig eluted with PBS.

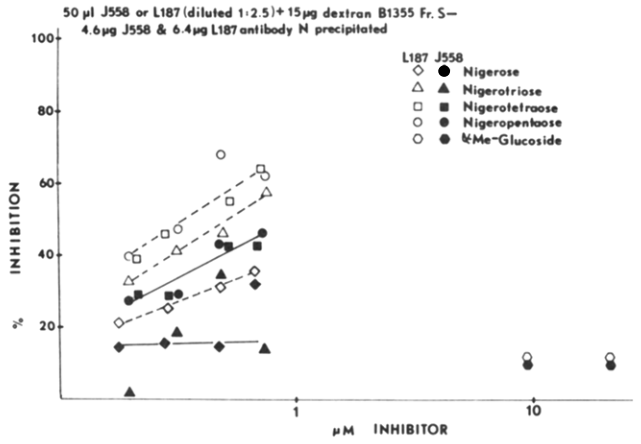

FIGURE 3: Inhibition by various oligosaccharides of precipitation of myeloma proteins J558 and L187 with dextran B1355 S. Precipitin curves were performed with myeloma protein from the ascitic fluid from CDF_1 mice (BALB/c × DBA) that had been injected intraperitoneally with J558 or L187 cells. The amount of antibody nitrogen was determined by the ninhydrin reagent. A point on the precipitin curve at 80% precipitation in slight antibody excess was chosen for the inhibition studies. At this point, 4.6 µg of J558 and 6.4 µg of L187 antibody nitrogen were precipitated by dextran B1355 S. Inhibitors were added in the indicated quantities, and the percentage inhibition of precipitation was determined.

tetraose, and both were better inhibitors than the smaller oligosaccharides, consistent with the published observations for J558 (Lundblad et al., 1972). These data suggested that

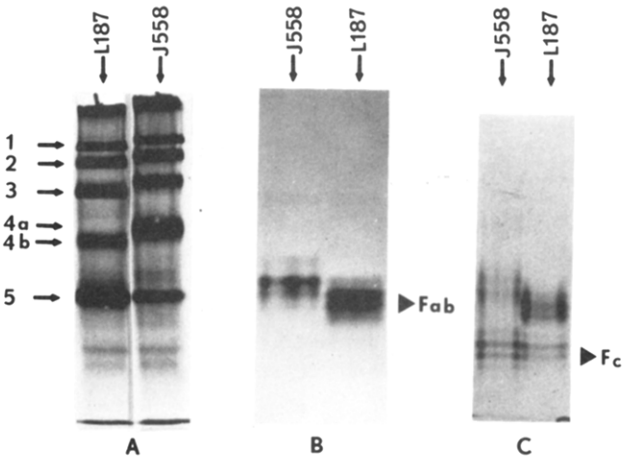

FIGURE 4: Migration of secreted Ig and Fab and Fc fragments from L187 and J558 on Tris–glycine gels containing 5% polyacrylamide and no $NaDodSO_4$. Cells were incubated for 24–36 h in media containing ^{14}C-labeled valine, threonine, and leucine and the supernatants recovered and dialyzed against 0.06 M Tris-HCl, pH 6.9, prior to electrophoresis. Fab and Fc fragments were isolated after cleavage with papain and separated on DEAE-Sephadex A-25. (A) Intact Ig; (B) Fab fragments; (C) Fc fragments with contaminating Fab. The identification of the Fab fragments was verified by adding polymeric dextran to the gels; the migration of the Fab fragments was inhibited (not shown). Bands 1–3, polymers of Ig H_2L_2; band 4, H_2L_2; band 5, free L chain.

the size of the L187 combining site was very similar to that of the J558 site. However, on a molar basis, the oligosaccharides were more effective inhibitors of the precipitation of L187 Ig with dextran B1355 than of the precipitation of J558 Ig with dextran B1355 (Figure 3). This difference is especially noticeable with nigerose and nigerotetraose, which over the concentrations used were effective inhibitors of L187 but poor inhibitors of J558. This result implied that the reaction between L187 Ig and polymeric dextran was weaker than that between J558 and polymeric dextran, and therefore, it was easier to inhibit the binding of L187 Ig to polymeric dextran using the oligosaccharides. Thus, the antigen binding mutant L187 maintains approximately the same site size and pattern of reactivity with small oligosaccharides as its parent, J558, but has decreased reactivity with polymeric dextran.

Examination of the Immunoglobulin Produced by L187 and J558. The structural changes in the L187 Ig responsible for the alterations in the ability to bind antigen were identified. L187 produced H and L chains indistinguishable on $NaDodSO_4$–polyacrylamide gels from those of J558 (data not shown). However, when the Igs from L187 and J558 were examined on Tris–glycine–polyacrylamide gels that did not contain $NaDodSO_4$, a difference in their migration pattern was seen (Figure 4A, bands 1–4). Bands 1–5 (Figure 4A) were recovered from these gels, and their compositons were determined by analysis on $NaDodSO_4$–polyacrylamide gels before and after disruption of disulfide bonds. Bands 1–3 contained polymeric IgA, $(H_2L_2)_n$, with $n_1 > n_2 > n_3$. Band 4 contained $(H_2L_2)_1$. Band 5, which migrated identically in mutant and wild-type cells, contained free L chain. As in all BALB/c IgAs, there were disulfide bonds between the H chains, but association of H and L chains was through noncovalent interactions. Bands 1–4 were inhibited from migrating in the gel when dextran was added to the gel, verifying that they contained Ig that bound dextran (Takeo & Kabat, 1978). The migration of band 5 was unaffected by the addition of dextran. Since Tris–glycine gels without $NaDodSO_4$ separate proteins as a function of both their charge and their molecular weight (M_r), and there was no apparent difference

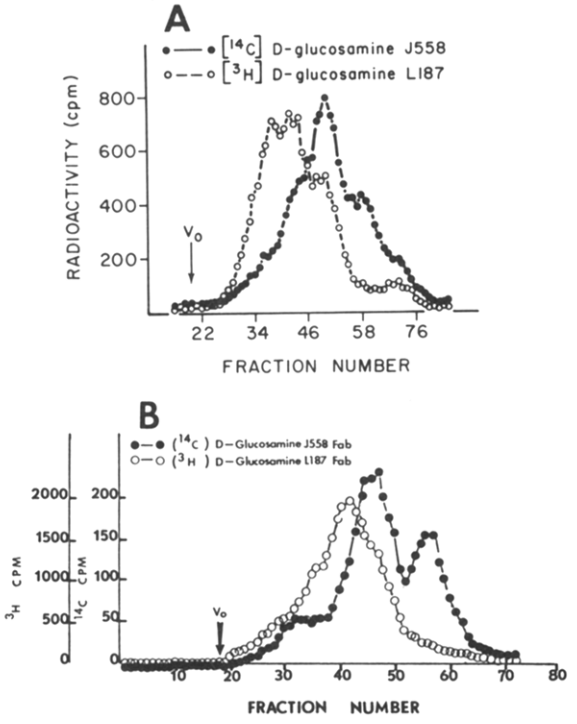

FIGURE 5: Analysis of glycopeptides from the H chains of J558 and L187 on Bio-Gel P6 columns. Ig was labeled with either D-[^{14}C]- or D-[^{3}H]glucosamine hydrochloride by incubating cells for 48 h in medium containing the radioactive sugar and recovering the secretions. Following reduction and alkylation, H chains were purified on Sephadex G150 columns equilibrated in 8 M urea and 0.1 M formic acid. Fab was prepared as described in Figure 4. Fractions were desalted on G25 columns in 0.05 M formic acid, lyophilized to dryness to remove the formic acid, and digested for 48 h with Pronase under a layer of toluene. Fresh Pronase (1 mg) was added at 0, 6, 24, and 36 h. Glycopeptides were chromatographed on Bio-Gel P6 columns (1.5 × 150 cm) equilibrated in 0.05 M NH_4HCO_3. Blue dextran and phenol red were used to mark the excluded and included volumes. Fractions (1.5 mL) were collected, and the positions of the glucosamine-containing glycopeptides were determined by counting aliquots of each fraction in a liquid scintillation counter. The elution volume of glycopeptides on Bio-Gel columns depends on the size of the carbohydrate; larger carbohydrates are eluted in a smaller volume.

in M_r between the H and L chains of L187 and J558 indicated by $NaDodSO_4$ gels, the differences in migration observed in Figure 4A must be due to differences in charge.

When the Fab fragments from the L187 and J558 were compared (Figure 4B), a difference in migration was seen while no difference was seen when the Fc fragments were compared (Figure 4C). These data show that L187, a mutant altered in its ability to bind dextran, has a charge difference in the Fab region of the Ig. [^{14}C]VTL-labeled Ig lacking CHO was prepared by growth of cells in high levels of glucosamine and examined on Tris–glycine gels without $NaDodSO_4$. J558 and L187 migrate similarly (data not shown), indicating that the migration difference was due to a difference in the carbohydrate content of the two Igs.

Analysis of Glycopeptides of L187 and J558 Igs. The carbohydrate on J558 and L187 Igs was labeled by incubating the cells in media containing [^{3}H]- or [^{14}C]glucosamine, and labeled H chains were purified from cell secretions. H chains were digested exhaustively with pronase to remove protein and the remaining glycopeptides examined on Bio-Gel P6 columns. The profile obtained when glycopeptides from J558 labeled with [^{14}C]glucosamine and those from L187 labeled with [^{3}H]glucosamine were examined is shown in Figure 5A. The majority of glycopeptides derived from L187 H chains appear larger than those from J558 H chains. A similar elution

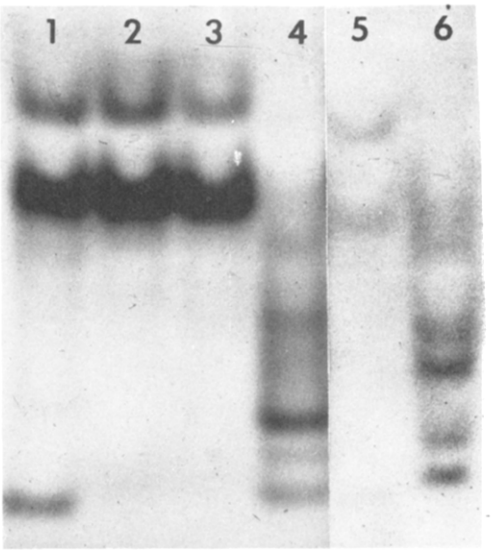

FIGURE 6: Analysis of glycopeptides from L187 and J558 Igs on Tris–borate–10% polyacrylamide gels. Secreted Ig that was labeled with [^{14}C]glucosamine was isolated from the culture supernatants of L187 and J558 cells following papain cleavage; Fab was isolated by binding to a dextran–BSA–Sepharose affinity column and elution with 0.1 N glycine, pH 2.2. Intact Ig was immunoprecipitated from culture supernatants with rabbit anti-J558 Ig followed by sheep anti-rabbit Ig. Igs were digested with Pronase in 0.05 M NH_4HCO_3, pH 8.0, for 48 h under a layer of toluene. After digestion with Pronase, the 0.05 M NH_4CO_3, pH 8.0, was removed by lyophilization. For neuraminidase digestions, glycopeptides dissolved in 0.05 M NH_4HCO_3 were adjusted to pH 5.5 with acetic acid and digested with 5 μg of enzyme at 37 °C for 16 h. Samples were lyophilized; the glycopeptides were dissolved in 0.01 M Tris–borate, pH 8.7, and analyzed on Tris–borate–polyacrylamide gels. The gels were dried directly onto filter paper, and the positions of the peaks containing glucosamine were determined by autoradiography. Lane 1, J558; lane 2, J558 + neuraminidase; lane 3, L187 + neuraminidase; lane 4, L187; lane 5, J558 Fab; lane 6, L187 Fab.

pattern was observed when the glycopeptides derived from Fab fragments of J558 and L187 Igs were compared (Figure 5B).

[^{14}C]Glucosamine-labeled glycopeptides derived from L187 were found to migrate more rapidly on Tris–borate gels than those from J558 (Figure 6). Carbohydrates bind different amounts of borate ions, depending on their size and composition; larger carbohydrates and carbohydrates containing sialic acid migrate more rapidly. This difference in migration was also observed when glycopeptides from the Fab fragment were compared (Figure 6), verifying the difference in glycosylation of the Fab fragment. One fast-moving glycopeptide was common to both L187 and J558 (marked by arrow) but was absent from the Fab fragment, indicating that it was most probably located on the Fc fragment. After digestion with neuraminidase, L187 glycopeptides migrated at the same rate as the two major wild-type glycopeptides (Figure 6). Thus, the glycopeptides of L187 contain varying amounts of sialic acid. The two major glycopeptides of J558 were unaffected by digestion with neuraminidase, indicating that they did not contain sialic acid. However, the fast-moving carbohydrate present on both L187 and J558 and probably located in the Fc region disappeared after neuraminidase treatment, indicating that it contained sialic acid. Thus, it appears that the glycopeptides of the mutant H chain contain more sialic acid than do the glycopeptides from the J558 H chain. However, the wild-type H chain does not appear to be entirely devoid of sialic acid.

Peptide Map Analysis. The structures of the mutant and wild-type proteins were compared by peptide mapping of tryptic peptides. L187 and J558 Igs were labeled with arginine

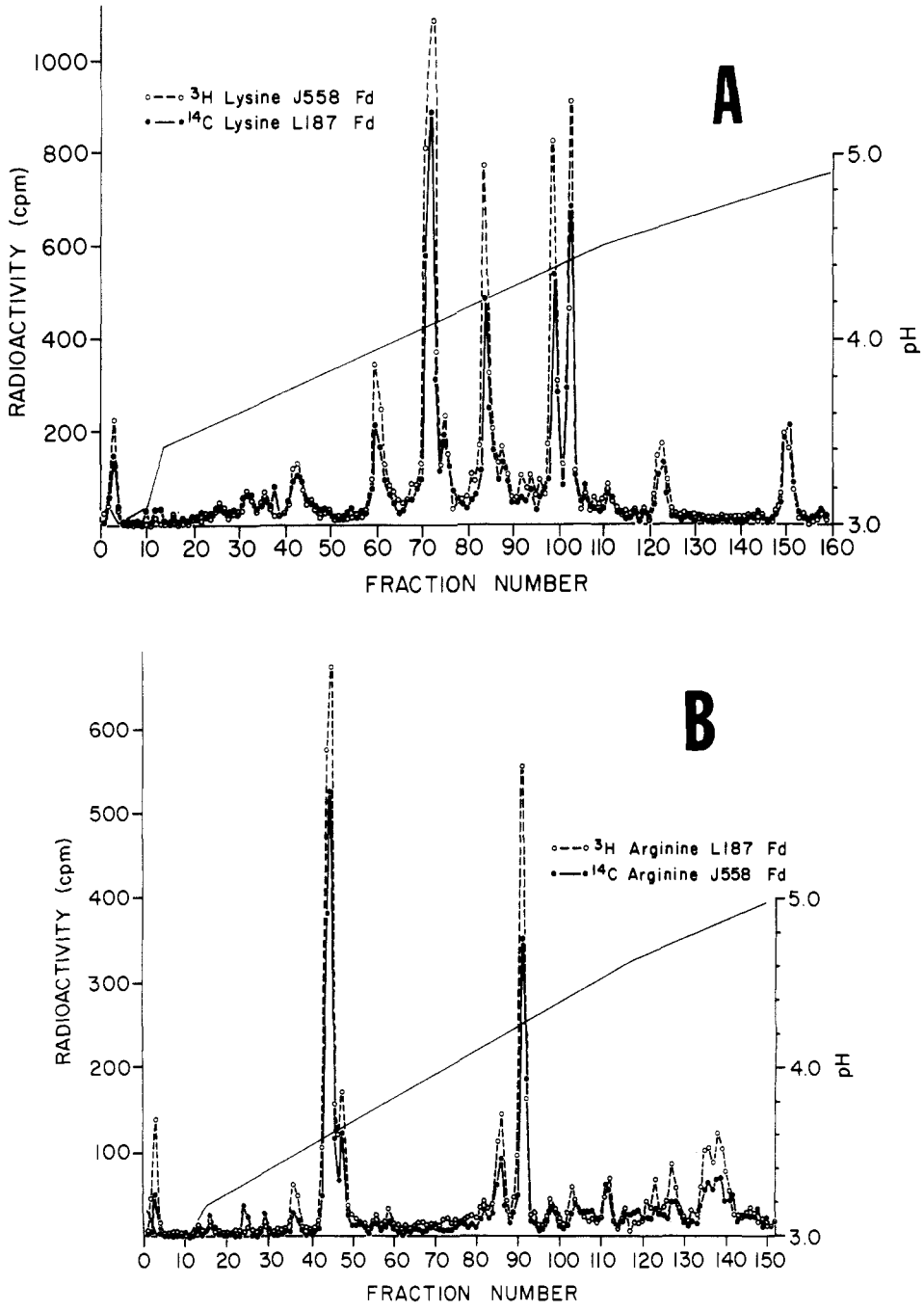

FIGURE 7: Tryptic map profiles of arginine- and lysine-labeled Fd from J558 and L187 cells. Cells were incubated with radioactive arginine or lysine, and the secreted Ig was isolated by immunoprecipitation with rabbit anti-J558 and SAR. The precipitates were digested with papain to generate Fab and Fc, completely reduced with 0.3 M β-mercaptoethanol in 2% $NaDodSO_4$ for 1 h at 37 °C, and applied to cylindrical $NaDodSO_4$–phosphate–polyacrylamide (5%) gels. Gels were electrophoresed at 60 V for 16 h, after which the gels were crushed into 4-mm pieces, and the Ig in each slice was eluted with 1% $NaDodSO_4$. Aliquots of each fraction were counted in a liquid scintillation counter, and the positions of the peaks containing labeled arginine or lysine were identified. Under these conditions, L chain and Fd and Fc fragments were separated and recovered from gel slices. Purified Fd was precipitated with cold 10% trichloroacetic acid and prepared for peptide map analysis as described under Materials and Methods. (A) [^{3}H]Lysine-labeled J558 Fd versus [^{14}C]lysine-labeled L187 Fd; (b) [^{3}H]arginine-labeled J558 Fd versus [^{14}C]arginine-labeled L187 Fd.

or lysine, the L chain and Fd and Fc fragments were isolated, and the peptide map profiles of tryptic peptides were compared. In Figure 7, the peptide maps of the L187 and J558 Fd fragment labeled with arginine or lysine are shown. In addition, Ig without carbohydrate labeled with lysine was prepared, and the peptide map profile of the tryptic peptides of the Fd fragment was compared. No consistent differences were seen by using the technique of peptide mapping. However, carbohydrate-containing peptides are not well resolved by this method, and peptides with a carbohydrate moiety and a change in charge probably would go undetected.

Examination of the Glycosyltransferases Available in Mutant and Wild-Type Cells. If there was a difference in the amino acid sequence of the mutant, it could have gone undetected by peptide mapping. However, one simple hypothesis to explain the difference in the carbohydrate content of the two Igs is that there are different glycosyltransferases in the mutant and wild-type cells. To test the hypothesis that the glycosyltransferase content of L187 led to differences in glycosylation of the H chain and altered reactivity with polymeric antigen, we performed two experiments: (1) Mutant and wild-type cells were infected with a virus (VSV) and the

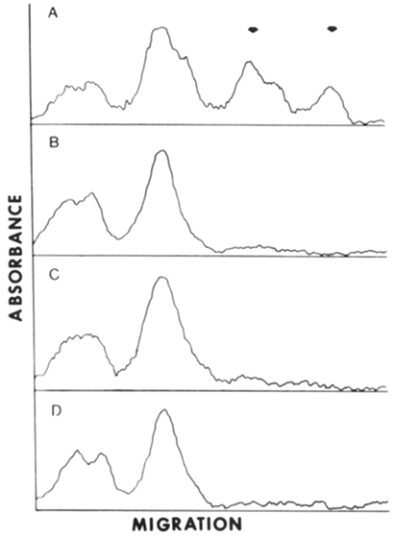

FIGURE 8: Densitometer scans of the Tris–borate electrophoresis pattern of glycopeptides isolated from vesicular stomatitis virus (VSV) glycoprotein G. Viral proteins labeled with [^{14}C]glucosamine were recovered from infected J558 and L187 cell lysates by immunoprecipitation with anti-VSV and SAR. Precipitates were digested with Pronase and analyzed on Tris–borate gels before and after treatment with neuraminidase as described in Figure 6. Glycoprotein G from VSV grown in L187 (lanes A and B) and J558 (lanes C and D). Glycopeptides shown in lanes B and D were treated with neuraminidase. Rapidly migrating glycopeptides containing sialic acid are indicated by the arrows.

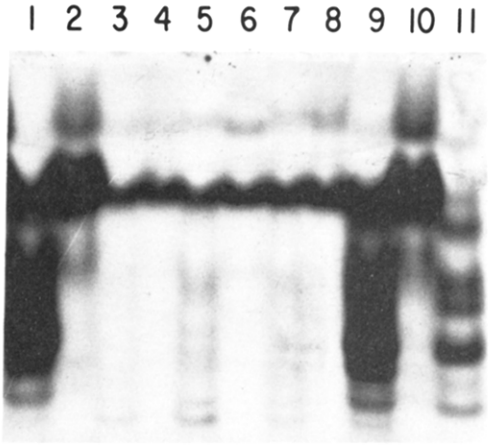

FIGURE 9: Migration on Tris–borate–polyacrylamide gels of glycopeptides derived from Ig secreted from J558, L187, 4T001, J514, and J603 cells. J558 and L187 Igs labeled with [^{14}C]glucosamine were precipitated with anti-IgA and SAR. 4T001 Ig was precipitated with anti-IgG_{2b} Fc and SAR. Ig in hybrids (J514 and J603) was first precipitated with rabbit anti-IgG_{2b} Fc and SAR, and then the supernatants were reprecipitated with rabbit anti-IgA and SAR. Control experiments indicated that anti-IgA precipitated only IgA in the hybrid and that anti-IgG_{2b} Fc only precipitated IgG_{2b}, suggesting there were no mixed molecules. Precipitates were digested with Pronase and analyzed before and after digestion with neuraminidase (N). (1) J514 IgA; (2) J514 IgA + N; (3) J514 IgG_{2b}; (4) J514 IgG_{2b} + N; (5) 4T001; (6) 4T001 + N; (7) J603 IgG_{2b}; (8) J603 IgG_{2b} + N; (9) J603 IgA; (10) J603 IgA + N; (11) L187. J514 is the 4T001 × J558 hybrid and J603 is the 4T001 × L187 hybrid.

carbohydrates on the viral glycoprotein obtained from these cells compared. A difference in the enzyme complement of the cells might be reflected in the carbohydrate of the virus since the virus relies on host-cell enzymes for glycosylation (Moyer & Summers, 1974; Etichison & Holland, 1974). (2) Somatic cell hybrids between L187 or J558 and a drug-marked derivative of 45.6 (4T001) were made and the carbohydrate and antigen binding capacities of the Ig in the hybrids compared.

L187, J558, and BSC40 cells infected with vesicular stomatitis virus (VSV) were incubated in medium containing [^{14}C]glucosamine; the viral proteins were precipitated with guinea pig anti-VSV and examined before and after neuraminidase treatment on Tris–borate gels. As seen in Figure 8, glycoprotein G from virus grown in L187 (lane A) contained carbohydrate which migrates rapidly on Tris–borate gels (marked by arrows). After treatment with neuraminidase, these rapidly migrating glycopeptides are not seen (Figure 8, lane B). There was no evidence for sialic acid on glycoprotein G of VSV grown in J558 cells (lanes C and D). Glycoproteins G from VSV grown in BSC40 cells contained sialic acid (data not shown). These results indicated that L187 cells contained enzymes which facilitated the sialylation of both L187 Ig and glycoprotein G; J558 cells did not contain this enzyme.

For further examination of the glycosyltransferase content of the myeloma cells, somatic cell hybrids were made between J558 and 4T001 (IgG_{2b}, κ) and between L187 and 4T001. Hybrids contained H and L chains from both parents, and all four chains were found in the secretion of these cells. In both J603, the hybrid between L187 and 4T001, and J514, the hybrid between J558 and 4T001, the IgA-derived glycopeptides (lanes 1, 2, 9, and 10) contained sialic acid. The IgG_{2b} heavy chain from 4T001 contained small amounts of sialic acid in both the parental and the hybrid cells (lanes 3–8). These data indicated that (1) although J558 Ig was usually deficient in sialic acid the IgA of wild-type J558 could be heavy sialylated in the proper cellular environment and that (2) J558 did not have an inhibitor of glycosylation but instead lacked glycosyltransferases. The glycopeptide patterns of the IgA H chains of both hybrids, J514 (J558 derived) and J603 (L187 derived), were very similar, indicating that in the same environment these two chains could be equivalently glycosylated (Figure 9, lanes 1 and 9).

If the difference in the antigen binding capacity of the L187 and J558 proteins results solely from different degrees of glycosylation, two predictions are made: (1) L187 and J558 proteins which contain no carbohydrate should bind antigen equivalently, and (2) L187 and J558 proteins which contain the same high levels of carbohydrate should bind antigen to the same degree. Efforts were made to isolate L187 and J558 heavy chains free of carbohydrate. Neither L187 nor J558 intact Ig was susceptible to cleavage by neuraminidase or endoglycosides. When J558 or L187 cells were grown in the presence of high levels of glucosamine to inhibit glycosylation, the heavy chains secreted by these cells were found to be highly susceptible to cleavage by proteases, and no analysis of its binding capacity was possible.

However, both the J558 (J514) and L187 (J603) Igs produced in the somatic cell hybrids with 4T001 were found to contain equivalent levels of sialic acid and to be stable. When the antigen binding capacity of these Igs was examined with radioimmunoassay, the results shown in Figure 10 were obtained. In an environment in which they apparently contained the same amount of carbohydrate, L187 and J558 proteins bound antigen to an equivalent degree and less well than wild-type J558 protein which contained little sialic acid.

Additional Antigen Binding Mutants. Additional independent mutants were isolated from J558 that showed altered reactivity with dextran. When these mutants were labeled with [^{14}C]glucosamine and the glycopeptides analyzed on Tris–borate gels, a heterogeneous group of glycopeptides like those observed for L187 was observed. Treatment of the glycopeptides with neuraminidase resulted in alteration of their migration, indicating that they contained sialic acid. It is not

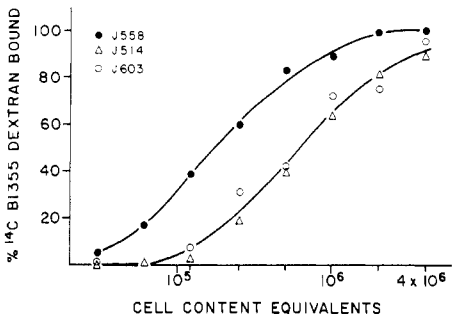

FIGURE 10: Binding of ^{14}C-labeled B1355 S and L dextran by cytoplasmic Ig from J558, J514, and J603 cells. Varying amounts of cytoplasmic Ig were added to a constant amount of [^{14}C]dextran, and the assay was performed as described in Figure 1. The cytoplasmic Ig content of the cells was determined; no differences were found between the amount of IgA contained in the cytoplasms of J558, J514, and J603 cells. Each point represents an average of two determinations.

certain if all antigen binding mutants contained exactly the same sialylated glycopeptides as L187; however, it is clear that the glycopeptides contain more sialic acid than those from J558 Ig. Thus, in several independently arising mutants, our findings also showed a decreased reactivity with polymeric dextran associated with an alteration in the pattern of glycosylation of the H chains.

Discussion

In the experiments described in this paper, we have used the J558 mouse myeloma cells as a model system to study the expression of Ig by plasma cells and to investigate alterations in antibody structure which lead to changed reactivity with antigen. The J558 Ig shares both light chain type and idiotypic determinants with other myeloma proteins [e.g., MOPC 104 (IgM, λ; anti-α1→3 dextran)] (Weigert et al., 1970; Cesari & Weigert, 1973; Hansburg et al., 1977, 1978), with hybridomas specific for α1→3 dextran (Schilling et al., 1980), and with antibodies in the serum of BALB/c mice immunized with dextran (Blomberg et al., 1972). The Ig that J558 synthesizes is thus representative of the antibody repertoire which is normally expressed in the immunized mouse. The amino acid sequences of the V_H regions of J558, MOPC104, and the hybridoma anti-α1→3 antibodies have been determined, and the majority of the sequences from position 1 to 99 of the H chain are identical. Carbohydrate has been found at position 55 in the second variable region of the H chain of J558, MOPC 104, and of nine of the ten hybridoma antibodies that have been sequenced (Kehry et al., 1979). X-ray diffraction studies of the antibody combining sites of the anti-PC protein M603 have placed amino acid number 55 of the H chain at the lip of the antibody combining site (Segal et al., 1974). The J558 combining site has not been examined by X-ray diffraction, but amino acid 55 would be expected to be at an analogous position in the J558 combining site. Carbohydrate is also probably present at residue 155 of the CH_1 domain of the H chain (Robinson & Appella, 1977, 1979) and residue 419 of CH_3 (Robinson & Appella, 1980).

We have isolated a mutant of J558 producing an Ig altered in its ability to react with polymeric dextran. The carbohydrate on the mutant Ig was found to contain more sialic acid than did the carbohydrate of the wild-type Ig. The relative order of reactivity of a series of oligosaccharides was the same in L187 and J558; thus, the extra addition of sialic acid on the carbohydrate apparently does not change the size of the antibody combining site; instead, it probably weakens reactivity with polymeric antigens by steric or charge effects. The alteration observed in the mutant is not strictly an alteration in specificity but instead is an alteration in binding of determinant groups when attached to a large molecule.

Differences in carbohydrate structure on the Ig could be due to alterations in the primary structure that affect glycosylation or due to alterations in the complement of glycosyltransferases of the cell synthesizing the Ig. No differences in the primary structure of the Ig could be detected by peptide mapping when Fd or Fc fragments or L chains from mutant and wild types were compared. This, however, does not eliminate the possibility of differences in amino acid sequence that are not detected by this method.

In contrast, a clear difference between the complement of available glycosyltransferases in mutant and wild-type cells could be shown by comparing the glycosylation of the same protein (VSV glycoprotein G) in different cellular environments (J558, L187, or BSC40 cells). Glycoprotein G synthesized in L187 and BSC40 cells contained sialic acid while the glycoprotein G synthesized in J558 cells did not contain sialic acid. Additional evidence for the availability of different glycosyltransferases in different myeloma cell lines was obtained by using somatic cell hybrids. In somatic cell hybrids with MPC-11 (4T001), both the IgA of J558 and of L187 contained large amounts of sialic acid. These results indicate that the α chain of J558 can accept sialic acid in the proper cellular environment and that the lack of sialylation in J558 is a recessive and not a dominant phenotype. L187 and J558 proteins synthesized in the somatic hybrids were found to bind antigen similarly and less well than the wild type. Since somatic cell hybridization should not change the amino acid sequence of the protein, this finding supports the idea that altered glycosylation leads to altered reactivity with antigen.

It remains to be shown whether alterations in glycosylation are important in vivo in generating altered antibody–antigen reactivity. Additional independently arising mutants of J558 have been shown to have altered reactivity with polymeric dextran and, like L187, to have glycopeptides containing additional sialic acid, suggesting that the lesion(s) resulting in altered glycosylation is (are) not a rare event. Studies of BALB/c hybridomas synthesizing IgA molecules specific for α1→3 or α1→6 dextrans have shown that those specific for α1→3 dextran contain sialic acid while those specific for α1→6-linked dextran contain little to no sialic acid (L. Matsuuchi, J. Sharon, and S. L. Morrison, unpublished experiments). Therefore, J558 itself may be a mutant that occurred in vivo and was selected for because of enhanced binding of polymeric antigen; L187 would then be an in vitro revertant. The antigen binding alteration in L187 is decreased reactivity with polymeric antigen; it should be noted that most biologically important antigens exist in vivo not as free hapten but associated with larger carrier proteins or cell surfaces. In addition, the antigen binding variants isolated from S107 are phenotypically similar to L187; that is, they bind haptens as well as the parental cell line but do not react as well as the parental cell line with hapten attached to carrier (M. D. Scharff, unpublished experiments).

Somatic mutants of eukaryotic cells with defects in the ability to glycosylate proteins have been identified previously and include mutants that survive the toxic effects of plant lectins and contain alterations in their glycosylating ability (Gottlieb & Kornfeld, 1976; Stanley et al., 1975). Previously defined abnormalities in the glycosylation of Igs are associated with Ig chains of altered primary structure (Weitzman et al., 1977, 1979a). On the other hand, L187 has no demonstrable changes in the structure of its heavy chains, but does have clear changes in its glycosyltransferase content. Study of the gly-

cosylation of α chains in J558 and L187 and in the somatic cell hybrids provides one with the opportunity to study the glycosylation of the same protein in different cellular environments. As yet, such studies have been limited to viral proteins. Igs have the advantage that they are produced in large quantities, and the secondary effects of viral infection on the cell need not be considered. Study of variation in glycosylation among Igs also provides a means of assessing the effect of different carbohydrate structures on protein assembly, secretion, and membrane insertion, and for antigen binding proteins, interaction with antigen.

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