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Preliminary crystallographic data for an Fab to the melanoma-associated GD2 ganglioside, and the purification of a soluble form of this antigen.

By SUSAN L. PICHLA, RAMACHANDRAN MURALI and ROGER M. BURNETT, *The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104, USA*

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Abstract

An Fab fragment from a monoclonal antibody (ME36.1) to the melanoma-associated GD2 ganglioside has been purified and crystallized in space group $P2_1$ with unit-cell dimensions a = 37.6, b = 94.1, c = 67.4 Å, β = 101.0°. The crystals, which grow to a size of up to 0.6 × 0.5 × 0.3 mm, diffract to 2.5 Å and native data have been collected to 2.8 Å resolution. The crystal density is 1.22 g ml^{-1} indicating one molecule of 48 kDa per asymmetric unit and a solvent content of 51%. A soluble form of the carbohydrate was obtained from the scarce GD2 glycolipid by enzymatic digestion with ceramide-glycanase. Small co-crystals of the Fab–GD2 complex have been obtained. As ME36.1 has been used in immunotherapy to treat malignant melanoma, knowledge of its interactions with the ganglioside could increase the efficacy of these treatments.

Introduction

As the use of antibodies in disease detection and treatment increases, the need to understand the specific interactions between the antibody and its antigen becomes extremely important. Insight into the different types of binding interactions could create new ways of attacking specific diseases. Knowledge of these interactions would effect the way the disease is targeted and how it is treated.

One area of increased interest in using antibodies to treat disease is the use of antitumor antibodies in immunotherapy for cancer (Lubeck *et al.*, 1985). In particular, antibodies to melanoma cell-associated antigens have been used to identify the different stages of the progression of the cancer and to treat the disease (Herlyn *et al.*, 1985). These different stages display different profiles of cell-surface carbohydrates that can be identified by specific anticarbohydrate antibodies. The sialic acid cell-surface glycolipids, or gangliosides, are the most drastically changing functional groups on the melanoma cell surface (Hakomori, 1985). Normal melanocytes express GM3 as the most prominent cell-surface ganglioside. As the neoplastic transformation commences, the distribution and type of expressed gangliosides starts to change. In the case of melanoma, this is due to a triggering of glycosyltransferases that glycosylate GM3 to form gangliosides such as GD2 and GD3. These gangliosides are normally expressed at low levels but are highly expressed on malignant melanoma cells (Thurin *et al.*, 1987). The accumulation of GD2 and GD3 on the melanoma cell surface appears to be correlated with the proliferation of the cancer (Ravindranath, Tsuchida & Irie, 1989).

The monoclonal antibody ME36.1 recognizes the GD2 ganglioside (Thurin *et al.*, 1987) and has shown potential for use in immunotherapy (Iliopoulos *et al.*, 1989). Although the function of the GD2 ganglioside is still unclear, it is believed to be involved in cell-to-cell signaling or cell-to-substrate interactions (Ravindranath *et al.*, 1989). The structure of the Fab–GD2 complex could serve as a model for these interactions and would give information about the structural properties of the GD2 carbohydrate that have been very difficult to obtain so far. Most importantly, independent structures of the native Fab and the complex would show if any conformational changes of the antibody occur upon binding. This information could lead to drugs that mimic the interactions, or to the modification of the antibody by means of genetic engineering to increase its therapeutic value.

Materials and methods

Fab fragments were prepared from pure samples of ME36.1 antibody, which were provided by E. Merck (Darmstadt, Germany). The optimal conditions for the digestion of the antibody were determined by using various ratios of papain to antibody over various lengths of time. Each digest was analyzed using non-reducing sodium dodecyl sulfate and isoelectric focusing (IEF) gels (Pharmacia Phastsystem). The monoclonal antibody was digested at 310 K for 1 h with papain (Sigma) at a 1:100 (*w*/*w*) ratio of enzyme to antibody in a solution containing 100 m*M* NaAc (pH 5.5), 1 m*M* dithiothreitol and 2 m*M* EDTA. The digestion was stopped with the addition of an excess of antipain (Boerhinger Manheim) to papain, where approximately 2 µg of antipain inhibits 1 unit of papain (1 unit hydrolyzes 1.0 µ*M* of benzyl arginine ethyl ester per min at pH 6.2 and 298 K). The Fab was separated from the Fc by running the digest at pH 7.6 through a Mono S cation-exchange column on a Fast Performance Liquid Chromatography system (Pharmacia). The Fc eluted in the flowthrough, and the Fab was eluted from the column with a 0–1.0 *M* NaCl gradient. Analysis of the eluted Fab by isoelectric focusing showed three distinct Fab isoforms. These were separated on a Mono Q anion-exchange column by running a shallow pH gradient from pH 9.5 to 7.0 (Garcia, Ronco, Verroust & Amzel, 1989). Analysis of the peaks using IEF gels showed complete separation of the isoforms.

Co-crystallization with the GD2 antigen was dependent on obtaining a soluble form of the antigen. This was carried out by cleaving the ceramide group from the antigenic carbohydrate portion of GD2 (GalNAcβ1→4Gal[3←2αNeuNAc8←2αNeuNAc]β1→4Glcβ1→1Cer) by digestion with ceramide glycanase (Li & Li, 1989). 12 vials, each containing 90 µg of lyophilized GD2 ganglioside, were used in the digest. The GD2 ganglioside (E. Merck, Darmstadt, Germany) was solubilized by adding 300 µg of sodium choleate to each vial and then digested with 0.5 units of ceramide glycanase for 16–18 h at 310 K in NaAc buffer (pH 5.0). The digest was stopped with a chloroform:methanol (2:1) solution and the aqueous phase containing the soluble carbohydrate was separated from the organic phase. Any contaminating hydrophobic components remaining in the aqueous phase were removed by running the sample through a hydrophobic C_{18} Sep-Pak column (Millipore). Thin-layer chromatography was used to demonstrate the purity of the final soluble carbohydrate.

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A solid-phase radio immunoassay (RIA) was used to test the binding of the purified Fab to the GD2 ganglioside. The glycolipid was serially diluted in ethanol and applied to a 96-well microtiter plate (Dynatech Laboratories, Alexandria, VA) in 50 µl well volumes and allowed to dry overnight at room temperature. The primary antibody (ME36.1 Fab) was then added at 20 µg ml^{-1} and incubated for 2 h at room temperature. Binding of the primary antibody was detected with an ^{125}I-labeled goat anti-mouse H+L antibody (1000 counts min^{-1} µl^{-1}) (Jackson Laboratories) as the second antibody.

Results and discussion

Crystals of the Fab fragment of ME36.1 (Fig. 1) were obtained by vapor diffusion using the hanging-drop method (McPherson, 1982). The Fab crystallized in 3–4 d as orthogonal parallelepipeds, which grew to dimensions up to 0.6 × 0.5 × 0.3 mm in 30%(*w*/*v*) PEG 4000, 1% 2-methyl-2-4-pentanediol (MPD) and Tris buffer at a pH of 7.5. The crystals belong to the monoclinic space group $P2_1$ with unit-cell dimensions of a = 37.6, b = 94.1, c = 67.4 Å and β = 101.0°. Density measurements (Matthews, 1974) indicated that there is one Fab molecule in the asymmetric unit with a V_m of 2.49 Å^3 Da^{-1} and a solvent content of 51%.

Six separate diffraction data sets were collected from native crystals on a Siemens–Nicolet X100-A multiwire area detector. The X-ray source was a Rigaku RU200 rotating-anode generator, equipped with a double-mirror focusing system (Supper) and operated at 40 kV and 65 mA. A 15 cm crystal-to-detector distance was used with 0.20 frames (ω steps) and exposures of 5 min. The data sets were reduced and merged using *XDS* (Kabsch, 1988) to give a unique set with an R_{sym} of 10.1%. The final data set was 95.4% complete to 2.8 Å resolution. A plot of the *h*0*l* plane of the reciprocal lattice is shown in Fig. 2. The Fab portion of the J539 Fab structure (Suh *et al.*, 1986) has been used as a search model to determine the orientation and position of the ME36.1 Fab in the unknown crystal environment, and structure solution is proceeding.

Crystallization trials of the complex at a 4:1 molar ratio of GD2 to Fab using the same crystallization conditions as the native Fab were unsuccessful. A fresh screen of conditions (Crystal Screen, Hampton Research) was used to find those favorable for co-crystal growth. Clusters of long needles grew in 20%(*w*/*v*) PEG 4000, sodium citrate at pH 5.6 and 20%(*v*/*v*) 2-propanol. Slight modifications of these conditions produced thin plate-like crystals. Further refinement of these conditions to grow crystals suitable for structural analysis is continuing. An attempt was made to produce a crystalline complex by soaking the carbohydrate into native Fab crystals. Addition of small molar quantities of the carbohydrate over several days produced visible etching on the surface of these crystals. The crystals soaked with GD2 also lasted longer in the X-ray beam and diffracted to a slightly higher resolution than the unsoaked crystals. The presence of the carbohydrate in the antigen-binding site of these soaked crystals will be investigated with difference-density maps.

The interest in using antibodies in immunotherapy has led to attempts to humanize mouse monoclonal antibodies that have shown therapeutic potential. Humanized antibodies tend to be less immunogenic (Kettleborough, Saldanha, Heath, Morrison & Bendig, 1991). Attempts at humanizing the ME36.1 antibody by CDR grafting the antigen-binding loops onto a human variable region can result in the loss of antigen binding. It has been observed that the framework residues surrounding the CDR loops are important for the correct folding of these loops (Chothia *et al.*, 1989). Knowledge of the Fab structure of ME36.1 would help to determine which residues surrounding the antigen-binding loops contribute to their conformation. These residues could then be mutated in the human variable region so as to maintain the correct conformation of the CDR graft.

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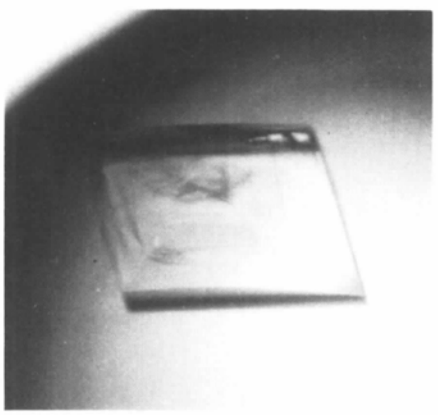

Fig. 1. A single crystal of native ME36.1 Fab grown from 30%(*w*/*v*) PEG 4000, 1% MPD and Tris buffer at pH 7.5 with dimensions 0.6 × 0.5 × 0.3 mm. The space group is monoclinic $P2_1$.

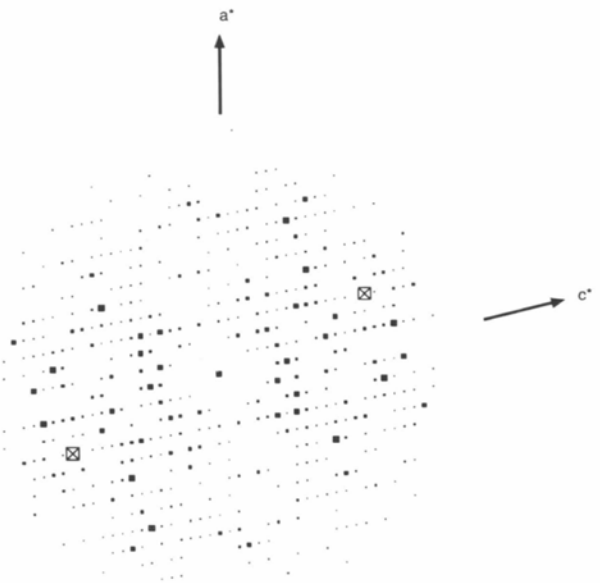

Fig. 2. A pseudoprecession plot of the *h*0*l* zone extending to 2.5 Å resolution calculated using the *HKLPLOT* program from the *CCP*4 program suite (SERC Daresbury Laboratory, 1979). The angle between the a^* and c^* reciprocal axes (β^*) is 79°.

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