Analytical Biochemistry 411 (2011) 271–276

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Measurement of serum levels of natalizumab, an immunoglobulin G4 therapeutic monoclonal antibody

Theo Rispens [a,b,*], Astrid van Leeuwen [c], Anke Vennegoor [d], Joep Killestein [d], Rob C. Aalberse [a,b], Gerrit J. Wolbink [a,b], Lucien A. Aarden [a,b]

[a] Sanquin Research, 1066 CX Amsterdam, The Netherlands
[b] Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands
[c] Sanquin Diagnostic Services, 1066 CX Amsterdam, The Netherlands
[d] Department of Neurology, VU University Medical Center, 1081 HV Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 8 November 2010
Received in revised form 6 December 2010
Accepted 1 January 2011
Available online 7 January 2011

Keywords:
IgG4
Half-molecule
Immunoglobulin
Fc interactions
Therapeutic antibody
Natalizumab
ELISA

ABSTRACT

Human immunoglobulin G4 (IgG4) is a poor trigger of effector functions and, therefore, is the preferred subclass for therapeutic monoclonal antibodies that merely aim to block their in vivo targets. An example is natalizumab, a recombinant IgG4 antibody directed against α4-integrin and used for treatment of multiple sclerosis. Efficient treatment requires that the pharmacokinetics of therapeutic monoclonal antibodies can be accurately monitored. For natalizumab, this requires special precautions due to recently reported structural peculiarities of human IgG4. Here we describe the development of an assay to determine serum levels of natalizumab. Compared with other IgG subclasses, human IgG4 possesses unique structural properties that influence its interactions in both in vivo and in vitro settings. Thus, IgG4 undergoes Fab arm exchange in vivo, resulting in effectively monovalent antibodies. Furthermore, IgG4 is able to bind to other human and nonhuman IgG via Fc interactions. We demonstrate how these features can interfere with measurement of specific IgG4 and describe how we addressed these issues, resulting in an assay that is not sensitive to Fab arm exchange by natalizumab or to IgG4 Fc interactions.

Introduction

Immunoglobulin G4 (IgG4)[1] is the least abundant of the human IgG subclasses, comprising approximately 4% of the total IgG in serum. However, antigen-specific antibody levels may be dominated by IgG4, usually in situations of chronic or repeated antigenic challenge [1]. The correlation of allergen-specific IgG4 with beneficial effects of immunotherapy suggests that IgG4 is tolerogenic in nature [2]. Indeed, IgG4 is the least effective IgG antibody in terms of effector function, with diminished affinities to most Fc gamma receptors and lacking the ability to activate complement [3,4]. Furthermore, in vivo, IgG4 typically is effectively monovalent due to Fab arm exchange, resulting in antibodies that can block formation of (large) immune complexes [5–8]. Therefore, in a therapeutic setting, IgG4 may be selected to serve as blocking antibody, largely avoiding triggering of effector mechanisms.

An example is natalizumab, a recombinant antibody directed against α4-integrin used for treatment of multiple sclerosis (MS). Natalizumab selectively inhibits α4-integrin-mediated adhesion of lymphocytes to endothelial receptors (vascular cell adhesion molecule 1 and mucosal addressin cell adhesion molecule 1), thereby preventing migration across the blood–brain barrier into the central nervous system (CNS). Natalizumab is derived from a murine monoclonal antibody humanized by complementarity-determining region (CDR) grafting onto a human IgG4 framework [9].

Efficient treatment using therapeutic monoclonal antibodies such as natalizumab requires that serum drug levels can be accurately determined, for example, to optimize dosing regimens. Furthermore, patients treated with natalizumab may develop progressive multifocal leukoencephalopathy (PML) [10,11]. The outcome of PML depends mainly on fast and complete removal of the drug using, for example, plasma exchange. Obviously, decisions on whether or not plasma exchange is needed to wash out the drug may also depend on timely measurements of natalizumab concentrations, and detailed pharmacokinetic studies are needed.

In the case of natalizumab, measurement of drug levels requires special precautions due to structural peculiarities of human IgG4. As noted, IgG4 will undergo Fab arm exchange in vivo, and it

* Corresponding author at: Sanquin Research, 1066 CX Amsterdam, The Netherlands. Fax: +31 20 512 3170.
E-mail address: t.rispens@sanquin.nl (T. Rispens).

[1] *Abbreviations used:* IgG4, immunoglobulin G4; MS, multiple sclerosis; PML, progressive multifocal leukoencephalopathy; HRP, horseradish peroxidase; IVIG, intravenous immunoglobulin; GSH, reduced glutathione; ELISA, enzyme-linked immunosorbent assay; HPE, high-performance ELISA; RF, rheumatoid factor.

0003-2697/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2011.01.001

was shown that natalizumab will also participate in the exchange process [12]. Furthermore, IgG4 is able to bind to other human and nonhuman IgG [13–15], complicating the setup of a reliable specific assay. Here we describe the development of an assay to determine serum levels of a therapeutic monoclonal IgG4 antibody. We demonstrate that the above-mentioned factors do indeed complicate development of a quantitative assay. An assay format that takes into account the special features of IgG4 and can be used to accurately measure natalizumab in serum is presented.

Materials and methods

Materials and reagents

Natalizumab (Tysabri, Biogen Idec and Elan Pharmaceuticals) was supplied as a 20-mg/ml liquid. This formulation also contains sodium chloride, sodium phosphate, and polysorbate 80. Other humanized monoclonal antibodies used were omalizumab (Xolair, Novartis) and efalizumab (Raptiva, Genentech, Merck Serono). Anti-IgG (MH16-1–horseradish peroxidase [HRP] conjugate) and anti-IgG4 (MH164.1– and MH164.4–HRP conjugate) mouse monoclonal antibodies were obtained from Sanquin Reagents (Amsterdam). MH164.1 was biotinylated with Sulfo-NHS LC-Biotin (Pierce/Thermo Fisher Scientific, Rockford, IL, USA). Recombinant chimeric mouse/human antibodies to *Fel d* 1 were produced as described previously [6]. IgG4 paraprotein was kindly provided by Sanquin Reagents. Sera were obtained from healthy volunteers. Serum samples from individuals with relapsing MS treated with natalizumab were drawn under informed consent after approval of the ethics committee of the VU University Medical Center (Amsterdam). Patients received natalizumab (at a dose of 300 mg) by intravenous infusion every 4 weeks. Blood samples were obtained just before receiving a dose of natalizumab.

Pepsin digestion of natalizumab

Natalizumab (10 mg) was digested with pepsin (1:100, w/w) at pH 3.5 by overnight incubation at 37 °C. The reaction was stopped by adding 1 M Tris until the pH reached 7.5. The interchain form of natalizumab (which is the predominant isomer) results in the formation of F(ab)2 fragments, whereas the intrachain form will result in Fab fragments. After dialysis against PBS (10 mM sodium phosphate [pH 7.4] and 140 mM sodium chloride), the resulting Fab/F(ab)2 fragments were purified using a protein A Sepharose column (Pharmacia/GE Healthcare, Uppsala, Sweden) to remove traces of undigested material. Part of the purified material was sterile filtered and used for immunization (see below).

F(ab)2 and Fab fragments were separated by size exclusion chromatography. Samples (200 µl) were applied to a Superdex 200 HR 10/30 column (Amersham Biosciences, Uppsala, Sweden), which was connected to a high-performance liquid chromatography (HPLC) system (ÄKTA Explorer) from Amersham Biosciences. The column was equilibrated in PBS. Fractions were collected and pooled based on the elution profile (absorption at 280 nm [see Fig. 2C]). Both pools were concentrated using Amicon Centriprep centrifugal filter devices (Millipore, Billerica, MA, USA), and the second pool, corresponding to Fab fragments, was refractionated to remove residual F(ab)2 fragments. Protein concentrations were determined by measuring the absorbance at 280 nm with a NanoDrop ND-1000 spectrophotometer.

Polyclonal rabbit antibodies directed to the idiotype of natalizumab

Two female New Zealand white rabbits were immunized and boosted with 100 µg/ml natalizumab F(ab)2 fragments three times at 4-week intervals. Natalizumab F(ab)2 fragments were mixed with Montanide ISA-50 for each immunization. After the animals were sacrificed, serum was collected. IgG was purified from serum by protein A affinity chromatography (elution with 0.1 M glycine, pH 2.5) and dialyzed against PBS. Antibodies recognizing constant regions in the Fab domains of natalizumab were removed using an intravenous immunoglobulin (IVIG)–Sepharose column. To this end, 50 mg of IVIG-L (Nanogam, Sanquin Reagents) was coupled to 2500 mg of CNBr-activated Sepharose (Amersham Biosciences, Uppsala, Sweden) and packed into a column. The flow-through fraction was collected, and the procedure was repeated two more times. In between, the column was cleaned using an aqueous solution of 8 M urea and 1 M Tris at pH 8.0 to remove nonspecific

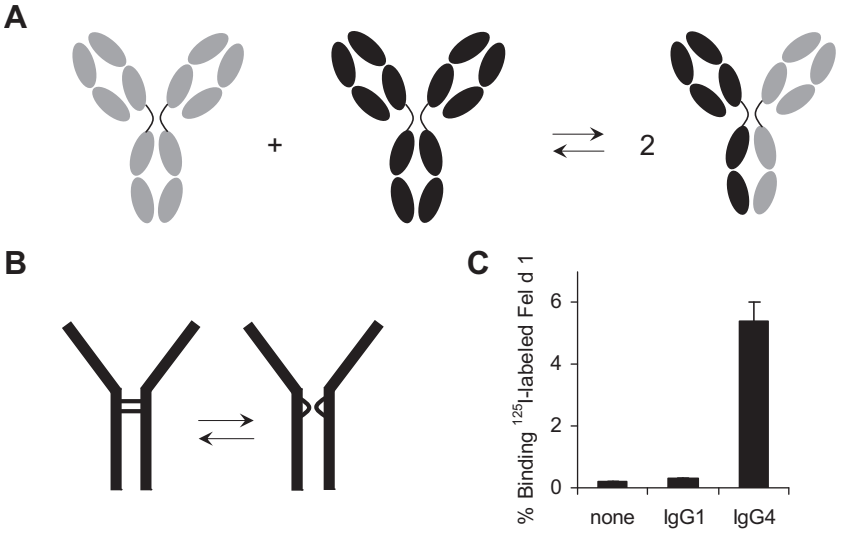

Fig.1. (A) Illustration of the exchange process of natalizumab leading to bispecific monovalent antibodies. The process appears to be random. Thus, for a given specificity (e.g., natalizumab), recombination with many other typically nonrelevant specificities will occur. Depending on the drug levels and total IgG4 levels, a smaller or larger part of the drug will become effectively monovalent. (B) IgG4 exists as two isomers, either with two hinge disulfide bonds connecting both heavy chains (interchain form) or with two intrachain disulfide bonds. (C) Exchange reaction of natalizumab. Natalizumab was incubated alone or with equimolar amounts of IgG1 and IgG4 anti-*Fel d* 1 for 24 h at 37 °C with 0.5 mM GSH. Bispecific cross-linking activity was detected using rabbit anti-natalizumab Sepharose and radiolabeled *Fel d* 1.