ELSEVIER

Journal of Immunological Methods 212 (1998) 187–192

JOURNAL OF IMMUNOLOGICAL METHODS

The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice

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Received 28 August 1997; revised 8 December 1997; accepted 6 January 1998

Abstract

Isotyping and quantitation of murine IgG2a antibodies are widely performed with commercial monoclonal and polyclonal antisera raised against BALB/c IgG2a myeloma proteins. Recently it became evident that inbred mouse strains with the *Igh1-b* allele do not have the gene for IgG2a and instead express the IgG2c isotype. We show that commercial anti-IgG2a sera cross-react inadequately against IgG2c in immunoblot and ELISA and hence, are not suitable to detect and measure this subclass in mouse strains such as C57BL/6, C57BL/10 and NOD. We have used DNA immunization to generate polyclonal anti-IgG2c serum and demonstrated that it is essential to use IgG2c-specific antiserum to quantify accurately isotypic responses in mouse strains with the *Igh1-b* allele.

Keywords: *Igh1* allele; Immunoglobulin isotyping; IgG2a; IgG2c

1. Introduction

Commercial monoclonal and polyclonal antisera to murine immunoglobulin (Ig) subclasses are widely used for isotyping and measuring the concentration of specific Ig subclass antibodies. The profile of Ig subclasses is of interest in analysing transgenic or knock-out mice and evaluating murine humoral responses to infections and vaccines. Furthermore, the switching to characteristic Ig subclasses is used to determine the polarisation of responses in the Th1/Th2 paradigm. In mice, antibodies of the IgG2a subclass are the predominant isotype produced to viral infections (e.g., Coutelier et al., 1987) and in cytokine-induced Th1-type responses (e.g., Snapper and Paul, 1987; Finkelman et al., 1988; Stevens et al., 1988).

The murine *Igh1-b* allele is the most divergent of the IgG2a heavy chains. The protein of this allele is distinctly different from other IgG2a allotypes by its elution profile from protein A (Seppälä et al., 1981) and by its unique allotypic determinants (Huang et al., 1986; Stall, 1996). However, it has become evident that the *Igh1-b* allele in mouse strains such as C57BL/6, C57BL/10, SJL and NOD is not an allele but is derived from a separate gene whose product is designated as the novel isotype IgG2c (Jouvin-Marche et al., 1989; Morgado et al., 1989; Martin et al., 1997). In these mouse strains the IgG2a gene is deleted, whereas in inbred strains with

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; Ig, immunoglobulin; PBS, phosphate buffered saline; RT, room temperature

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0022-1759/98/$19.00
PII S0022-1759(98)00015-5

the *Igh1-a* allele, like BALB/c, the IgG2c gene is deleted.

Commercial sera against the IgG2a isotype are raised exclusively against BALB/c IgG2a myeloma proteins. Because there is a 16% difference in the amino acid sequences between IgG2a and IgG2c, the question arises how reliably do anti-IgG2a sera react with IgG2c. The answer is critical for any interpretation of humoral immune responses in C57BL/6, C57BL/10, SJL and NOD mice. In this study, we demonstrate that conventional anti-IgG2a sera fails to detect or grossly underestimates levels of IgG2c.

2. Materials and methods

2.1. Detection of IgG2a by sandwich ELISA

Heavy chain specific, polyclonal goat anti-mouse IgG2a (1080-01; Southern Biotechnology, Birmingham, AL) or rabbit anti-mouse IgG2a (Z0014; DAKO, Carpinteria, CA) at 4 μg/ml in phosphate buffered saline (PBS) pH 7.2 was coated for 3 h at room temperature (RT) onto round bottom microtiter plates (Dynatech, Chantilly, VA) and blocked for 1 h at RT using PBS/5% skim milk. Serial dilutions of normal mouse serum or purified BALB/c IgG2a myeloma protein (Cappel, Organon Teknika, Durham, NC) in PBS/5% skim milk were incubated overnight at 4°C. Bound immunoglobulins were detected following incubation for 3 h at RT using polyclonal horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG2a (1080-05; Southern Biotechnology) diluted 1/5000 in PBS/5% skim milk. The volume used for each step was 50 μl and plates were washed three times with PBS/0.05% Tween 20 between all steps. After developing in tetramethylbenzidine solution, the enzyme-linked immunosorbent assay (ELISA) was terminated by the addition of 50 μl of 1 M H_2SO_4 and quantified at 450 nm using a BioTek Instruments UV900HDi ELISA plate reader.

2.2. Generation of anti-IgG2c serum by DNA immunization

The IgG2c heavy chain was cloned from genomic DNA of NOD mice as described previously (Martin et al., 1997). The IgG2c constant region comprising the hinge, CH2 and CH3 was subcloned as a fusion with a leader sequence for secretion in the expression vector pCIGH which contains a CMV/intron promoter, a murine CTLA4 targeting sequence and bovine growth hormone poly A signal. Plasmid preparation and targeting strategy has been described previously (Boyle et al., 1997, 1998). To generate antisera, BALB/c mice were injected intradermally with 50 μg of plasmid and boosted intradermally at 6 weeks with 50 μg plasmid. Mice were bled at 8 weeks and the sera analyzed by ELISA.

2.3. Detection of IgG2c by sandwich ELISA

Plates were coated with anti-IgG2a sera and incubated with normal mouse sera (from various strains) as described under Section 2.1. Polyclonal anti-NOD IgG2c (1/5000 in PBS/5% skim milk) was used as the secondary antiserum for 3 h at RT. Bound secondary antibodies were detected as described in Section 2.1 but using HRP-conjugated goat anti-mouse IgG1 (1070-05; Southern Biotechnology; 1/5000 in PBS/5% skim milk).

2.4. Isotype characterization using commercial assays

The ELISA-based Mouse Typer® sub-isotyping panel (172-2055; Bio-Rad Laboratories, Hercules, CA) and the agglutination based IsoStrip™ mouse isotyping kit (1493-027; Boehringer Mannheim, Indianapolis, IN) were used to identify mouse immunoglobulin class and sub-class in serum from NOD and BALB/c mice. Assays were performed according to the manufacturers' protocols.

2.5. Affinity purification of IgG2c

Immunoglobulins from NOD mouse sera were bound to protein A Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) at pH 7.2. IgG2a and IgG2c were eluted from protein A at pH 4.0 with 0.1 M citrate buffer and pH neutralized with 1 M Tris pH 9.5. The differential purification was required to avoid contamination with mouse IgG1 (mouse IgG1 binds poorly at pH 7.2; Ey et al., 1978), which

otherwise would be directly detected by HRP-conjugated goat anti-mouse IgG1 used as secondary antibody in immunoblots.

2.6. Immunoblot

Normal mouse serum (5 μl) was boiled for 5 min in 95 μl of 30% glycerol, 2% SDS, 10 mM β-mercaptoethanol, Tris–HCl (pH 6.5) and 10 μl electrophoretically separated on SDS 12.5%–polyacrylamide gels. Samples were electro-transferred to nitrocellulose sheets (Schleicher and Schuell, Dassel, Germany) in a semi-dry electrophoretic transfer cell (Pharmacia) according to the manufacturer's specifications and blocked for 1 h in PBS/5% skim milk powder. For the detection of IgG2a, membranes were incubated with either heavy chain-specific, HRP-conjugated goat anti-mouse IgG2a (1080-05; Southern Biotechnology; 1/5000 in PBS/5% skim milk) or heavy chain-specific rabbit anti-mouse IgG2a (Z0014, DAKO; 1/5000 in PBS/5% skim milk) followed by HRP-conjugated sheep anti-rabbit Ig (RAH; Silenus Laboratories, Victoria, Australia; 1/10,000 in PBS/5% skim milk). Total murine IgG was detected with HRP-conjugated goat anti-mouse IgG (6170-05; Southern Biotechnology; 1/5000 in PBS/5% skim milk). Affinity purified IgG2c was detected using anti-NOD IgG2c diluted 1/500 in PBS/5% skim milk, followed by incubation with HRP-conjugated goat anti-mouse IgG1 (Southern Biotechnology; 1/10,000 in PBS/5% skim milk). Membranes were washed three times with PBS/0.05% Tween 20 between all steps. Blots were developed using ECL (Amersham, Buckinghamshire, UK) and exposed to autoradiography film.

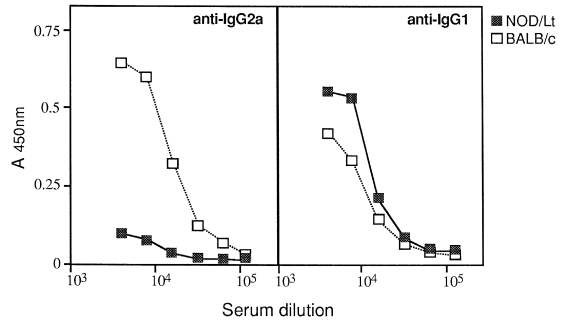

Fig. 2. Detection of IgG2a and IgG1 levels in NOD/Lt and BALB/c sera by ELISA using a commercial isotyping panel.

3. Results

3.1. Detection of IgG2a and IgG2c in mouse sera by sandwich ELISA

Commercial anti-IgG2a serum recognized IgG2a antibodies in BALB/c serum as well as purified IgG2a protein from a BALB/c myeloma, but it showed little crossreactivity with NOD and C57BL/10 sera (Fig. 1). Conversely, anti-serum raised to recombinant protein detected IgG2c in NOD and C57BL/10 sera, but did not react with BALB/c serum or purified IgG2a (Fig. 1).

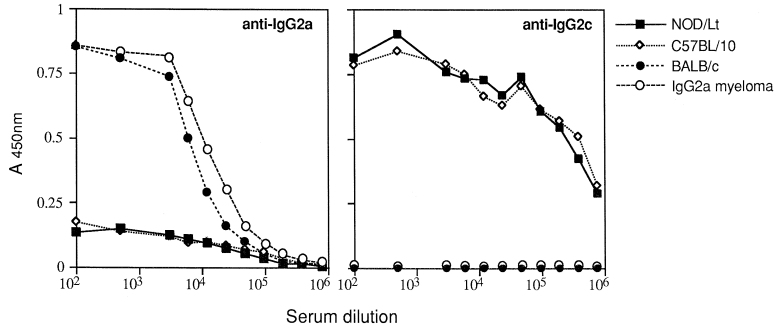

Fig. 1. IgG2a and IgG2c levels in sera from different mouse strains detected by sandwich ELISA using commercial anti-IgG2a or anti-IgG2c raised in BALB/c mice by DNA immunization.

3.2. Detection of serum IgG2c by commercial isotyping kits

Anti-IgG2a serum of the Mouse Typer® sub-isotyping panel detected IgG2a in BALB/c serum, but showed poor cross-reactivity to IgG2c in NOD serum (Fig. 2). Other antibody subclasses were detected at similar levels in serum from BALB/c and NOD mice (shown for IgG1 in Fig. 2). BALB/c serum (1/10,000 dilution) analysed with the IsoStrip assay resulted in a dominant band for the IgG2a subclass, while NOD serum used at the same dilution caused a faint band on the isotyping strip (data not shown).

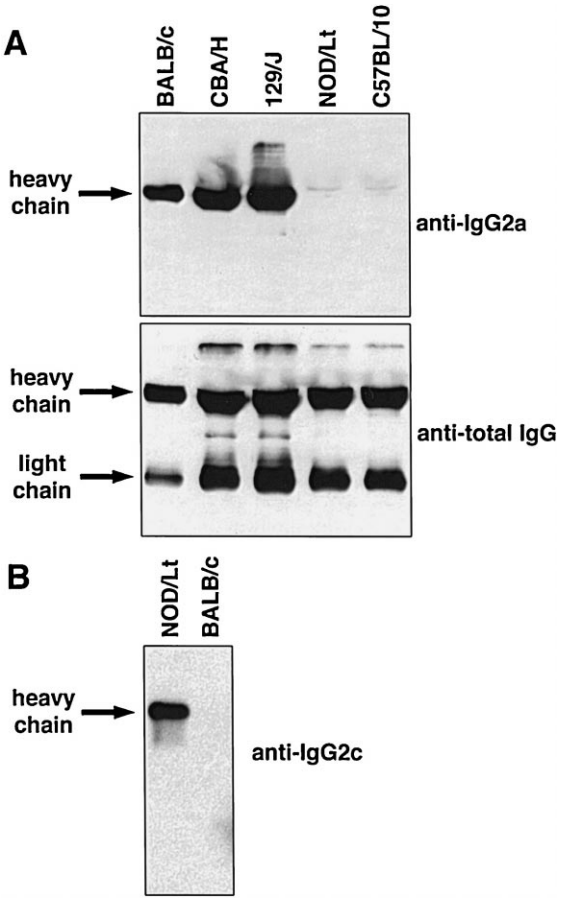

Fig. 3. (A) Detection of immunoglobulins in sera from different mouse strains by immunoblot using anti-IgG2a or anti-total IgG. Serum samples (equivalent to 0.5 μl serum) were run on SDS-PAGE under reducing conditions, transferred to nitrocellulose, probed with peroxidase conjugates and developed by chemiluminescence. (B) Detection of IgG2c after protein A purification by immunoblot. Protein A eluates at pH 4.0 from NOD/Lt and BALB/c sera were run on SDS-PAGE under reducing conditions, transferred to nitrocellulose and probed with anti-IgG2c raised in BALB/c mice by DNA immunization. Secondary mouse antibodies were detected with peroxidase conjugated goat anti-IgG1 sera and developed by chemiluminescence.

3.3. Detection of serum IgG2c by immunoblot

Commercial antiserum detected the heavy chain of the IgG2a isotype in sera of BALB/c, CBA/H and 129/J mice, but not NOD and C57BL/10 mice. As a positive control, antiserum to total IgG detected heavy and light chains in all mouse strains analyzed (Fig. 3A).

3.4. Detection of serum IgG2c purified on immobilized protein A

It has been demonstrated that IgG2a antibodies from mice of the *Igh-1b* allotype eluted at pH 4.5, while other allotypes eluted at pH 5.0 (Seppälä et al., 1981) consequently using differential pH (i.e., binding at pH 7.2 and elution at pH 4.0) both IgG2c or IgG2a would be eluted. Immunoblotting of purified IgG2a/IgG2c fractions showed that anti-IgG2c serum detected a band corresponding to heavy chains in the pH 4.0 eluate prepared from NOD serum, but not in BALB/c serum (Fig. 3B).

4. Discussion

The results of this study demonstrate that conventional immunoassays using antiserum against IgG2a are not suitable for analysis of antibody responses in mice with the *Igh1-b* allele, because of poor crossreactivity with IgG2c. This was found for four different commercial sources (Bio-Rad, Boehringer Mannheim, DAKO and Southern Biotechnology) in agglutination based strip tests, ELISA or immunoblotting. Therefore, it is essential to use IgG2c-specific antiserum in order to quantify accurately isotypic responses in C57BL/6, C57BL/10, NOD, and presumably other mouse strains with the *Igh1-b* allele. The use of specific IgG2c serum is also important with regard to isotyping monoclonal anti-

bodies derived in mice with the *Igh1-b* allele. It seems likely that, despite the poor crossreactivity of conventional isotyping kits, IgG2c would wrongly be subclassed as IgG2a, compromising antibody purification protocols and choice of an appropriate isotype control.

Seppälä et al. (1981) previously demonstrated that IgG2a of BALB/c eluted from protein A at pH 5.0, whereas the C57BL/6 equivalent eluted at pH 4.5. As elution of mouse IgG isotypes from immobilised protein A had long been known to be pH dependent (Ey et al., 1978), this should have alerted investigations to the possibility that the isotype of C57BL/6 was indeed different from BALB/c, especially given the genetic evidence of Jouvin-Marche et al. (1989). Cross-reactivity among different isotypes is generally low, even for hyperimmune sera (which is why the cross-reactive specificities can be absorbed out relatively easily).

Binding of IgG to protein A is mediated by the junctional region of the CH2 and CH3 domains of the heavy chain, with three sites being critical in this binding (Deisenhofer, 1981). For murine IgG2a these sites would be the MIS (140–142), QHQ (197–199) and NHH (322–324) positions described by Martin et al. (1997). It is interesting to note that there is only one residue that differs between IgG2a and IgG2c in these sites (H at position 324 is replaced by L) and this may be responsible for the different elution profile.

The question arises whether the exclusive expression of IgG2a or IgG2c reflects a yet unknown difference in the biological function of the two isotypes. Notably, studies of interferon-γ-stimulated IgG2a secretion by B lymphocytes (e.g., Snapper and Paul, 1987; Finkelman et al., 1988; Stevens et al., 1988) were done on mice other than those with the *Igh-1b* allele. Further studies are needed to clarify whether current knowledge of lymphokine-controlled isotype selection of IgG2a holds true for the IgG2c isotype. There are two situations where this is particularly relevant, namely in C57BL/6 mice that are resistant to leishmaniasis and in NOD mice that develop spontaneous insulin dependent diabetes. The presence or absence of high IgG2a levels has been proposed as a prototypic example of the Th1/Th2 paradigm in both examples (e.g., Locksley and Scott, 1991; Liblau et al., 1995).

Acknowledgements

We thank L.C. Harrison and J.W. Goding for critical review of the manuscript. This study was supported by a Diabetes Interdisciplinary Research Program Grant from the JDFI/NIH, USA; Apple Estate; Diabetes Australia and NHMRC Australia.

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