Journal of Immunological Methods 357 (2010) 26–32

Contents lists available at ScienceDirect

Journal of Immunological Methods

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

Research paper

A new ELISA plate based microtiter well assay for mycobacterial topoisomerase I for the direct screening of enzyme inhibitory monoclonal antibody supernatants

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ARTICLE INFO

Article history:
Received 30 December 2009
Received in revised form 6 March 2010
Accepted 9 March 2010
Available online 17 March 2010

Keywords:
Rapid screening
Microtiter plate
Monoclonal antibody
Topoisomerase I
Mycobacteria

ABSTRACT

Antigen specific monoclonal antibodies present in crude hybridoma supernatants are normally screened by ELISA on plates coated with the relevant antigen. Screening for inhibitory monoclonals to enzymes would require the evaluation of purified antibodies or antibody containing supernatants for their inhibition of enzyme activity in a separate assay. However, screening for inhibitory antibodies against DNA transacting enzymes such as topoisomerase I (topo I) cannot be done using hybridoma supernatants due to the presence of nucleases in tissue culture media containing foetal calf serum which degrade the DNA substrates upon addition. We have developed a simple and rapid screening procedure for the identification of clones that secrete inhibitory antibodies against mycobacterial topo I using 96 well ELISA microtiter plates. The principle of the method is the selective capture of monoclonal antibodies from crude hybridoma supernatants by topo I that is tethered to the plate through the use of plate-bound polyclonal anti-topo I antibodies. This step allows the nucleases present in the medium to be washed off leaving the inhibitor bound to the tethered enzyme. The inhibitory activity of the captured antibody is assessed by performing an *in situ* DNA relaxation assay by the addition of supercoiled DNA substrate directly to the microtiter well followed by the analysis of the reaction products by agarose gel electrophoresis. The validity of this method was confirmed by purification of the identified inhibitory antibody and its evaluation in a DNA relaxation assay. Elimination of all enzyme-inhibitory constituents of the culture medium from the well in which the inhibitory antibody is bound to the tethered enzyme may make this method broadly applicable to enzymes such as DNA gyrases, restriction enzymes and other DNA transaction enzymes. Further, the method is simple and avoids the need of prior antibody purification for testing its inhibitory activity.

1. Introduction

Monoclonal antibodies (mAbs) generated using the hybridoma technology (Kohler et al., 1976) are excellent tools in diagnosis, therapy and research (Waldmann, 1991). MAbs have also been invaluable in structure–function analysis of enzymes (Restle et al., 1992; Adami et al., 1993) and in studying protein–protein interactions (Ransone, 1995; Shi et al., 2000). In combination with other methods, inhibitory mAbs against enzymes have been used to probe reaction mechanisms and have given valuable insights into ligand/substrate-induced conformational changes in receptors as well as enzymes (Wakabayashi et al., 1986; Jiang et al., 2004; Jin et al., 2007; Gupta et al., 2008).

Abbreviations: MAbs, Monoclonal antibodies; PAb, Polyclonal antibody; Ms, *Mycbacterium smegmatis*; topo I, topoisomerase I.

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doi:10.1016/j.jim.2010.03.008

The generation of antigen specific mAbs involves the identification of antibody secreting clones that is normally done by screening hybridoma supernatants on ELISA microtiter plates coated with the relevant antigen. However, identification of enzyme inhibitory antibodies needs an additional step of testing the secreted mAb containing culture supernatants for inhibition separately. Hybridoma cultures are grown in Foetal Calf Serum (FCS) supplemented medium and the antibodies are secreted into the medium. Hence prior antibody purification would become necessary if culture supernatants interfere with the assay being performed. For example, the presence of nucleases in FCS (Cox and Gokcen, 1976; Wickstrom, 1986; Eder et al., 1991; Shaw et al., 1991) which degrade DNA would make it impossible to screen hybridoma supernatants for antibodies that inhibit DNA transacting enzymes such as topoisomerases. Prior antibody purification would become a pre-requisite in such situations but purification of hundreds of clones from large volumes of culture supernatants is a laborious and time-consuming task.

Although several methods have been described earlier for screening of antibodies, inhibitory mAbs and other inhibitors (Morgan et al., 1980; Rauch et al., 1985; Urban and Dreyfus, 1990; Ishii et al., 1997; Iwasa et al., 1983; Sivaraja et al., 1998; Cerretani et al., 1999; Mouratou et al., 2002; Chandrakala et al., 2004; Case et al., 2005; Chen et al., 2005; Ostrov et al., 2007; Ball et al., 1993) against various antigens using microtiter plates, no methods have been developed for the identification of mAbs that inhibit DNA transacting enzymes directly from the hybridoma supernatants. We have developed a simple and rapid screening procedure for screening inhibitory mAbs against mycobacterial topo I. The method employs the selective capture of mAbs from the culture supernatant by topo I that is, in turn bound to anti-topo I polyclonal antibodies (pAbs) adsorbed to the well surface. Nucleases and other contaminants were washed off in subsequent washing steps and the topo I assay was carried out *in situ* by adding supercoiled pUC18 DNA substrate. The effect of the mAbs was assessed by resolving the reaction products by agarose gel electrophoresis.

Topoisomerases maintain the topological homeostasis of cellular DNA (Gellert, 1981; Champoux, 2001) and their significance in normal cellular DNA metabolism is highlighted by the fact that several natural and synthetic topoisomerase inhibitors are used as antimicrobial and anticancer drugs (Drlica and Franco, 1988; Liu, 1989; Wang, 1994; Maxwell, 1997). The generation of a large repertoire of mAbs against *Mycobacterium smegmatis* topo I (Ms topoI) was described in our previous study which also showed that Ms topo I and *Mycobacterium tuberculosis* topo I (Mt topo I) were 88.9% similar (Leelaram et al., 2009). In this study we have employed this plate based microtiter well topo I assay to identify enzyme-inhibitory mAbs from the above repertoire of mAbs without their prior purification.

2. Materials and methods

2.1. Topo I mediated relaxation of supercoiled DNA

Topo I purification and activity assays were performed as described earlier (Bhaduri et al., 1998; Jain and Nagaraja, 2006) with minor modifications. Varying concentrations of topo I as indicated in the figure legends were pre-incubated with either unpurified or purified mAbs in assay buffer (40 mM Tris–Cl, pH 8.0, 20 mM NaCl and 5 mM $MgCl_2$) for 15 min on ice. The relaxation reactions were performed at 37 °C for 30 min with supercoiled pUC18 DNA by incubating at 37 °C for 30 min. The reaction products were resolved in 1.2% agarose gel at 1 V/cm for 12 h after the reactions were terminated with 0.1% sodium dodecyl sulphate (SDS) and heating at 75 °C for 10 min. The gels were stained with 0.5 μg/ml Ethidium Bromide (EtBr) and scanned using a Bio-rad gel documentation system. Y339F mutant topo I was generated by mega primer PCR method as described (Bhat et al., 2009).

When purified mAbs were evaluated for topo I inhibition, indicated amounts of topo I were pre-incubated with purified mAbs for 15 min on ice in 1.5 ml eppendorf tubes. Relaxation was performed at 37 °C for 30 min with 500 ng supercoiled pUC18 DNA substrate and the reaction products were analysed as described above. All the above assays have been referred to as a 'solution based tube assay' since the topo I mediated relaxation of DNA was carried out in 1.5 ml eppendorf tubes prior to the separation of relaxed products.

2.2. Microtiter plate topo I inhibition assay

Individual wells of a 96 well microtiter plate was coated with mouse anti-topo I antiserum at 1/500 dilution in assay buffer and incubated at 37 °C for 1 h. When pAbs purified from

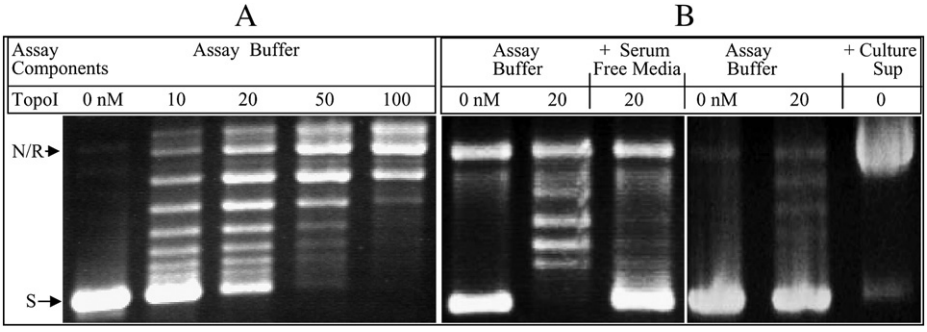

Fig. 1. Ms topo I is inactive in media with or without serum. Panel A. Topo I mediated DNA relaxation of supercoiled pUC18 DNA substrate was performed in eppendorf tubes with increasing amounts of purified topo I as given in Materials and methods. Panel B. Serum free media (15 μl) was added to the topo I reaction in a total volume of 20 μl as indicated while the addition of culture supernatant 5 μl to the supercoiled DNA substrate was done in the absence of topo I. The supercoiled (S) and relaxed (R) or nicked (N) forms of DNA are indicated by arrows on the left. Other bands indicate different topoisomers.