Regulatory Toxicology and Pharmacology 71 (2015) 141–147

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

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

Hep-2 cell based indirect immunofluorescence assay for antinuclear antibodies as a potential diagnosis of drug-induced autoimmunity in nonclinical toxicity testing

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ARTICLE INFO

Article history:
Received 13 January 2014
Available online 19 October 2014

Keywords:
Antinuclear antibodies (ANAs)
Age-related ANAs
Drug-induced autoimmunity (DIA)
Biomarker

ABSTRACT

Antinuclear antibodies (ANAs) are important biomarkers in the diagnosis of autoimmune diseases in humans; however, the diagnostic performance of ANA in nonclinical safety studies are not well understood. Here, we studied the use of ANAs as potential nonclinical biomarkers for drug-induced autoimmunity (DIA) using a Hep-2 based indirect immunofluorescence assay (IFA). Initially, MRL-fas^{lpr}/J mice and $HgCl_2$-treated rats were used as SLE-positive models. Serum samples obtained from 94 normal mice or 204 normal rats aged one to four months served as the negative control. The IFA effectively distinguished ANAs-positive samples in both species with a cut-off titer of 1:100. Brown Norway rats were treated with 450 mg/kg D-penicillamine for 30 consecutive days. ANAs were generated and corresponded with DIA development. Human Hep-2 cells, mice Neuro 2A cells, and Chinese Hamster Lung cells served as antigen from different species, which were found cross-reactive with ANA-positive serum samples from mice, rats, and humans without any differences in diagnosis. This methodology showed no species-specificity for ANA detection. Furthermore, we found approximately 20 percentage of the mice aged seven to eight months demonstrated age-related ANAs, which was consistent with humans. Overall, our findings demonstrated the use of ANA detection using IFA in the nonclinical diagnosis of murine drug-induced autoimmunity, and age-related ANAs should be considered when aged animals are used.

1. Introduction

Drug-induced autoimmunity (DIA) is recognized as one type of drug-induced immunotoxicity, such as drug-induced lupus, which clinically consists of 10% systemic lupus erythematosus (SLE) (Vedove et al., 2009). Currently, many biological products with immunological activities have been submitted to the regulatory authorities, and immune-related toxicity is specifically considered. Biopharmaceuticals, such as anti-TNF agents (infliximab, adalimumab, and etanercept) and interferon-alpha (Katz and Zandman-Goddard, 2010), vaccines (De Martino et al., 2013; Descotes et al., 2002) have been reported to trigger several autoimmune diseases, so patients are threaten in unexpected DIA. It is important to predict the potential of DIA in the preclinical phase evaluation of investigational new drugs (INDs).

Autoantibodies are one of the most important biomarkers in diagnosing clinical autoimmune disorders; they target local or global organs and tissues, and consequently cause organ-specific or systemic autoimmune diseases. Antinuclear antibodies (ANAs) consist of more than 100 autoantibodies (Perner et al., 2002), such as anti-double strand DNA antibody, anti-single strand DNA antibody, anti-histone antibody and anti-smith antibody. ANAs have been observed in many systemic autoimmune diseases; for example, ANAs have been detected in the serum of nearly 100% SLE patients (Mouritsen et al., 1986), 100% patients suffered with drug-induced lupus (Peng et al., 1997), and 90% patients with systemic sclerosis (Steen, 2005). Recently, studies showed that both sensitivity and specificity of the ANA in lupus diagnosis were more than 90% (Wichainun et al., 2013). Area under the curve of 0.916 was noted in ANA Hep-2 test (Brito Fde et al., 2014). Nowadays,

Abbreviations: ANAs, antinuclear antibodies; BN, Brown Norway; CHL, Chinese hamster lung; DIA, drug-induced autoimmunity; DP, D-penicillamine; FITC, fluorescein isothiocyanate; IFA, indirect immunofluorescence assay; SLE, systemic lupus erythematosus; PBST, PBS with 0.5% Tween 20.

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http://dx.doi.org/10.1016/j.yrtph.2014.10.005
0273-2300/

antinuclear antibody detection is still a necessary way to support DIA diagnosis (Xiao and Chang, 2014). Although these diseases should be confirmed using other clinical symptoms and signs. However, few studies on the methodology of ANA detection in nonclinical DIA diagnosis have been reported.

Despite being less validated, ANA detection using indirect immunofluorescence assay (IFA) has been used in murine serum samples for some time. ANAs were observed in mouse serum using formalinized chicken red cell nuclei as a nuclear antigen (Siegel et al., 1972; ten Veen and Feltkamp, 1972). ANAs in rat serum were also detected using a Hep-2 cell-based system (Al-Mogairen et al., 2009). These studies demonstrated that ANAs can be detected in murine serum, and their value for predicting and diagnosing DIA in nonclinical test may be valuable.

In the present studies, using human epithelial Hep-2 cell based slides in "EUROPLUS™ ANA Mosaic kits", we performed an indirect immunofluorescence assay (IFA) for ANA detection in murine models, and determined the cut-off titer in murine serum samples. To determine the relationship between ANAs and the development of autoimmune disease, D-penicillamine-induced lupus was induced in Brown Norway (BN) rats. Finally, the potential species-specificity of this assay and age-related ANAs in murine serum samples were determined.

2. Materials and methods

2.1. Reagents

EUROPLUS™ ANA Mosaic 20 (Hep-2 cells, primate liver) kits were obtained from EUROIMMUN (Hangzhou) Medical Laboratory Diagnostics Co., Ltd. (Hangzhou, Zhejiang, China). Both FITC-conjugated rabbit anti-mouse IgG (H&L) and FITC-conjugated rabbit anti-rat IgG (H&L) were purchased from Rockland Immunochemicals (Gilbertsville, Pennsylvania, US). $HgCl_2$ was provided and supervised by the National Institutes for Food and Drug Control (NIFDC). Chinese hamster lung (CHL) cells were preserved in the National Center for Safety Evaluation of Drugs (NCSED); Neuro 2A cells (a mouse neuroblastoma cell line) were kindly provided by Sinocelltech Ltd., Beijing, China; RMI1640 medium was provided by KeyGEN BioTECH Ltd. (Nanjing, Jiangsu, China); and fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, Zhejiang, China).

2.2. Animals care

All animals were bred in a specific pathogen-free facility at the NCSED and housed in polycarbonate cages containing corncob bedding at 20–26 °C, with 40–70% humidity and a 12-h light/dark cycle (07:00–19:00). NCSED is a Good Laboratory Principle facility authorized by the China Food and Drug Administration. Standard rodent diet and water were provided *ad libitum* throughout the study. There were no more than five animals per cage. The animal procedures were approved and supervised by the Institutional Animal Care and Use Committee (IACUC) of NCSED, the facility has been approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2.3. Establishment of murine SLE models

Five female MRL-fas^{lpr}/J mice, 21 weeks old, which suffered with SLE (Herkel et al., 2000; Wakeland et al., 1997; Dixon, 1982; Eisenberg, 1998) and were obtained from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). The MRL-fas^{lpr}/J mice from this center were confirmed suffering from SLE in previous researches (Guo et al., 2012; Luo et al., 2013). All mice were sacrificed after a five-day adjustment period, the blood was collected for serum preparation, and the kidneys were kept in formalin for pathological examination for further confirmation of this model.

Six Brown Norway rats were obtained from Vital River Laboratories (Beijing, China). After a three-day adjustment period, each animal was administered with 1 mg/kg $HgCl_2$ via subcutaneous injection five times within a 14-day period to induce lupus nephritis (Qasim et al., 1997; White et al., 2000; Dubey et al., 1991). Urinary proteins were examined using AM4290 urine analyzer at pre-dose on Days 7 and 14 of the experiment. On Day 15, all animals were sacrificed, and blood was collected for serum preparation.

2.4. Optimization of the IFA in the detection of murine ANAs

Serum samples from all MRL-fas^{lpr}/J mice were pooled and diluted at 1:100, 1:1000, and 1:5000 with PBST. Serum samples from $HgCl_2$-treated BN rats were prepared similarly as the mouse samples. The diluted serum samples were incubated with Hep-2 cells/liver smear on the Euroimmun slides (obtained from EUROPLUS™ ANA Mosaic 20 kits) for 30 min. Next, the slides were incubated with 1:100 FITC-conjugated rabbit anti-mouse IgG or rabbit anti-rat IgG after washing. Human ANA-positive serum and ANA-negative serum with FITC-conjugated sheep anti-human IgG served as the positive control and negative control, respectively. The slides were then screened using fluorescence microscopy after washing. Finally, non-pooled samples from individual animals were obtained for ANA detection as previously described. The diagnosis of ANA-positive and its pattern was based on fluorescence strength and location inside the Hep-2 cells determinate by a microscopy as described by Wiik et al. (2010).

To identify a cut-off titer for murine ANA detection, serum samples from 10 healthy Balb/c mice and twelve Wistar rats were tested at a dilution of 1:10 and 1:100, and 94 serum samples from healthy mice (Balb/c or Kunming) and 204 serum samples from healthy rats (Sprague Dawley or Wistar) were tested at a dilution of 1:100. Those healthy animals were non-treated or saline treated animals aged 1–4 months in other study performed in this facility without any abnormal findings in the parameters (if any) for toxicology evaluation such as clinical observation, hematological examination, chemical examination, or pathological examination. Serum samples were collection within five years and stored below −60 °C before ANA detection.

2.5. Development of ANAs in D-penicillamine-induced BN rats' lupus

Thirty male Brown Norway rats aged from six to seven weeks were randomly assigned into three groups: six rats were orally administered with deionized water and served as a negative control, twelve rats were dosed daily with 450 mg/kg/day D-penicillamine (DP) via P.O. route, and twelve rats were dosed daily with 450 mg/kg/day D-penicillamine plus PolyI:C (single dose prior to the first administration of D-penicillamine). From Day 8, blood samples were collected twice weekly to monitor ANA development. Six rats in both the D-penicillamine group and D-penicillamine + PolyI:C group were sacrificed when nearly all of the animals were ANA-positive. The remaining rats were dosed for up to 30 days and sacrificed thereafter. Heart, liver, lung, kidney, and mesenteric lymph nodes were collected, trimmed, dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H–E) for histopathologic evaluation by local pathologist. The development of DIA was confirmed by the lupus symptoms and microscopic examination.