FEBS Letters 581 (2007) 2017–2021

Anti-cytokine autoantibodies are ubiquitous in healthy individuals

Masato Watanabe[a,b], Kanji Uchida[c], Kazuhide Nakagaki[d], Hiroko Kanazawa[a], Bruce C. Trapnell[c], Yoshihiko Hoshino[e], Hiroshi Kagamu[a], Hirohisa Yoshizawa[a], Naoto Keicho[f], Hajime Goto[b], Koh Nakata[a,*]

[a] *Bioscience Medical Research Center, Niigata University Medical and Dental Hospital, Niigata 951-8520, Japan*
[b] *First department of Internal Medicine, School of Medicine, Kyorin University, Tokyo 181-8611, Japan*
[c] *Division of Pulmonary Biology and Neonatology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA*
[d] *Laboratory of Wildlife, College of Veterinary, Nippon Veterinary and Life Science University, Tokyo 180-8602, Japan*
[e] *Division of Pulmonary and Critical Care Medicine, Department of Medicine, New York University School of Medicine, New York, NY 10016, USA*
[f] *Department of Respiratory Diseases, International Medical Center of Japan, Tokyo 162-8655, Japan*

Received 17 February 2007; revised 3 April 2007; accepted 16 April 2007

Available online 24 April 2007

Edited by Masayuki Miyasaka

Abstract Anti-cytokine autoantibodies in healthy individuals have been widely reported but the occurrence is variable and inconstant. We hypothesized that cytokine-binding in vivo may explain their variable and infrequent detection. Therefore, we focused on the detection of the cytokine-autoantibody complexes and found that anti-cytokine autoantibody to IL-2, IL-8, tumor necrosis factor-α, vascular endothelial growth factor and granulocyte-colony stimulating factor were present in all 15 individuals evaluated, while those to IL-3, osteopontin and macrophage-colony stimulating factor were not detected in anyone. Autoantibodies against IL-4, IL-6, IL-10, and interferon-gamma were variously detected. Thus, we discovered that anti-cytokine autoantibodies to multiple cytokines are ubiquitous in healthy individuals.

Keywords: Autoantibody; Cytokine; Vascular endothelial growth factor; Immune complex; Healthy individual

1. Introduction

Anti-cytokine autoantibodies are present in a number of chronic inflammatory diseases [1,2], although a pathogenic role has not been clarified in most. In some instances, however, anti-cytokine autoantibodies are believed to be central to disease pathogenesis. For example, in some cases of Felty's syndrome, neutralizing autoantibodies against granulocyte-colony stimulating factor (G-CSF) appear to mediate neutropenia [3]. In pulmonary alveolar proteinosis, autoantibodies against granulocyte macrophage-colony stimulating factor (GM-CSF), which eliminate GM-CSF bioactivity in vivo, appear to cause alveolar macrophage dysfunction, resulting in impaired clearance of pulmonary surfactant, and respiratory insufficiency [4–6].

Anti-cytokine autoantibodies have been reported to be readily detected in some commercially available pharmaceutical intravenous human immunoglobulin (IVIG) preparations [7,8]. Anti-cytokine autoantibodies have also been detected, albeit rarely, in the sera of healthy individuals. For example, 1 in 300 individuals are positive for anti-cytokine antibodies by enzyme-linked immunosorbent assay (ELISA) [8] and 1 in 60 individuals are positive by radio-immuno assay [9]. The apparent discrepancy might be explained by inclusion of individuals with highly elevated levels of serum anti-cytokine autoantibodies in the donor pool used for preparing commercial immunoglobulin. Alternatively, anti-cytokine autoantibodies may be present at low levels in the serum of most healthy individuals, but in a poorly detectable form. In the present study, we hypothesized that anti-cytokine autoantibodies are highly prevalent in healthy individuals and that formation of cytokine autoantibody complexes in vivo impairs their detection by commonly used immunological methods based on ligand specificity. To elucidate this, we focused on the detection of cytokine-autoantibody complex by a newly developed method. We demonstrate the ubiquitous presence of anti-cytokine autoantibody in healthy individuals.

2. Materials and methods

2.1. Study subjects

Healthy individual was defined as one who did not have apparent past history of disease including cancer, collagen disease, and infection nor current episode of minor disease like common cold, allergy, and fatigue for one month. Every participants underwent health checkup annually. Eleven sera (male; $n = 5$, 30–51 y.o., female; $n = 6$, 20–49 y.o.), the three lots of fresh frozen plasma (FFP, Nisseki), and one lot of IVIG (Venoglobulin®-IH, Mitsubishi Pharma Corporation) were analyzed.

2.2. Dissociation of cytokine-autoantibody complexes

Serum/plasma or IVIG was fractionated with polyethylene glycol 6000 in order to precipitate both immune complex and free IgG, and to separate from free cytokines included in supernatant. The precipitate was re-suspended in 10 mM TBS (pH 8.0) and subjected to protein A/G column. Immunoglobulin G (IgG) was eluted with 100 mM

*Corresponding author. Fax: +81 25 227 0377.
E-mail address: radical@med.niigata-u.ac.jp (K. Nakata).

Abbreviations: G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor; IVIG, pharmaceutical intravenous human immunoglobulin; VEGF, vascular endothelial growth factor; $AutoAb_{VEGF}$, autoantibody against VEGF; TNF-α, tumor necrosis factor-α; Biotin-VEGF, Biotinylated recombinant human VEGF; K_{av}, average binding affinity; OPN, osteopontin; M-CSF, macrophage-colony stimulating factor; IFN-γ, interferon-gamma; ARDS, acute respiratory distress syndrome

0014-5793/$32.00
doi:10.1016/j.febslet.2007.04.029

glycine–HCl/0.15 M NaCl (pH 2.1) or ionic strength buffer (pH 6.55). The eluate was applied onto a spin column for ultrafiltration (Microcon® Centrifugal Filter Unit, Millipore, MW 100 kDa) and centrifuged at $6000 \times g$ in order to separate autoantibodies from autoantigens smaller than the 100 kDa. Recovered IgG were dialyzed against 10 mM TBS (pH 8.0). Recovered autoantigens were dialyzed against 10 mM TBS (pH 8.0) in the presence of bovine serum albumin (BSA), and concentrated with hygroscopic and high M.W. compound (PIERCE). This procedure for dissociation of cytokine-autoantibody complex was optimized by using vascular endothelial cell growth factor (VEGF)-monoclonal anti-VEGF antibody complex model as shown in supplementary data.

2.3. Isolation of autoantibody against VEGF (autoAb$_{VEGF}$)

AutoAb$_{VEGF}$ in acidified and size-separated IVIG (pH 2.1) was isolated with affinity chromatography coupled with rhVEGF, and eluted with 10 mM glycine–HCl/0.15 M NaCl (pH 2.1).

2.4. Immunoblotting

Cytokines (100 ng) were subjected to SDS–PAGE under reducing conditions and transferred to polyvinylidene fluoride membrane. The membrane was blocked and hybridized with acidified-ultrafiltrated IgG fraction for 2 h. Bound IgG was detected with HRP-anti-human IgG $(Fab')_2$ and visualized with ECL plus (GE Healthcare).

2.5. ELISA

Binding specificity of isolated autoAb$_{VEGF}$ was examined on ELISA plate pre-coated with cytokines, and determined by polymer-HRP-anti-human IgG. Inhibitory binding assay was carried out by incubating with recombinant human VEGF (rhVEGF) or recombinant human tumor necrosis factor-α (rhTNF-α) (final 2.4 μg/mL) prior to ELISA. IgG concentration of autoAb$_{VEGF}$ was measured by IgG assay kit (PIERCE).

The concentration of G-CSF or macrophage-colony stimulating factor (M-CSF) was also measured by ELISA kit (Biosource and R&D, respectively).

The occurrence of free anti-cytokine autoantibody in sera/plasma was determined by ELISA plate pre-coated with cytokines followed by detection with HRP-anti-human IgGF$(ab')_2$. When the OD value was higher than mean + 3S.D. of control level (cytokine not coated), the results were determined as positive.

2.6. Saturation binding assay

Biotinylated recombinant human VEGF (Biotin-VEGF) was made by NHS-PEO_4-Biotin (PIERCE). AutoAb$_{VEGF}$ or control IgG (humanized monoclonal anti-CD 20 antibody) was incubated with Biotin-VEGF (0–26.2 nM), each solution including Biotin-VEGF-autoAb$_{VEGF}$ complex was captured on ELISA plate pre-coated with anti-human IgG, and detected with AP-streptavidin, and CDP-Star® with Sapphire-II™ Enhancer (Applied Biosystems). Based on a Michaelis–Menten plot, average binding affinity (K_{av}) was determined from the concentration of free VEGF at 50% of the maximal binding.

3. Results

3.1. Occurrence of cytokine-autoantibody complexes in healthy individuals

We hypothesized that low or variable frequency in detection of the anti-cytokine autoantibodies in healthy individuals is due to the formulation of cytokine-autoantibody complex in vivo. To evaluate this, we focused on detection of the complex by a newly developed acidification-ultrafiltration procedure described in Section 2. We first evaluated the occurrence of anti-cytokine autoantibodies in commercially available IVIG preparations. Autoantibodies against VEGF, interleukin (IL)-2 and IL-8 were not detected in free IgG but were readily detected in the complex (Fig. 1A). We next evaluated the occurrence of the autoantibodies in serum/plasma from disease-free, healthy human individuals. Similar to the results with IVIG, autoantibodies against VEGF, IL-2 and IL-8 were readily detected in the complex but not in the free IgG (Fig. 1B). Then, autoantibodies directed at other cytokines were evaluated in the complex of serum/plasma of disease-free, healthy human individuals using a series of cytokine ligands for immunoblotting-based detection. Interestingly, all individuals tested had autoantibodies against multiple different cytokines (Fig. 1C, Table 1). Of the 15 individuals evaluated, all had autoantibodies against IL-2, IL-8, TNF-α, VEGF and G-CSF, 14 had autoantibodies to IL-4, 11 had autoantibodies to IL-10, and only 1 had antibodies to IL-6 or to interferon-gamma (IFN-γ). In contrast, no individuals had autoantibodies to IL-3, osteopontin (OPN), or to M-CSF either with or without acidification-ultrafiltration. None of the anti-cytokine autoantibodies evaluated were detected in the free IgG of serum/plasma samples (Table 1). These results demonstrate that anti-cytokine autoantibody is ubiquitously present in the form of cytokine-autoantibody complex in human serum and IVIG.

3.2. Binding specificity of autoAb$_{VEGF}$

To establish the specificity of anti-cytokine autoantibodies for their ligand targets, we first isolated autoAb$_{VEGF}$ from commercially available IVIG using the acidification-ultrafiltration step followed by VEGF-affinity chromatography. AutoAb$_{VEGF}$ was highly specific for VEGF (Fig. 2A), using a cytokine capture-based ELISA as we have previously use to demonstrate the specificity of anti-cytokine antibodies [6]. Further, soluble VEGF but not TNF-α significantly inhibited the detection of the autoantibody by ELISA (Fig. 2B). To further characterize the autoAb$_{VEGF}$, using a saturation binding plot approach, we determined the K_{av} of affinity-purified antibody to be 2.9 nM (Fig. 2C). Thus, autoAb$_{VEGF}$ from the serum of healthy individuals binds specifically to VEGF with moderate affinity.

3.3. Detection of bound ligand cytokines

While some cytokines (e.g., M-CSF) are readily detectable in serum, others (G-CSF) are not or are present in very low abundance. Hypothesizing that poorly detectable/low abundance serum cytokines may be present in the form of immune complexes and vice versa, we evaluated the acidified ultrafiltrate of human serum for the presence of M-CSF and G-CSF by ELISA. The level of detectable G-CSF in the serum ultrafiltrate was greatest when acidification was performed at a pH of 2.1 (Fig. 3A). Evaluation of serum from six individuals with and without acidification-ultrafiltration revealed that G-CSF in healthy human serum was completely bound to autoantibodies making it undetectable in a standard capture ELISA (Fig. 3B). Evaluation of M-CSF with and without acidification-ultrafiltration revealed that M-CSF was present in the serum in a form not bound by autoantibody (Fig. 3C). Thus M-CSF, a cytokine normally abundant in serum is not associated with anti-cytokine antibodies [10], while G-CSF, which is normally poorly/not detectable [11] is present in only in the form of complexes.

4. Discussion

Our results show that the detection of anti-cytokine autoantibodies in serum is hampered by binding to their respective