行政院國家科學委員會專題研究計畫　成果報告

研究 anti-ribosomal P 抗體之臨床價值與是否由黴菌感染所致

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全身性紅斑狼瘡以有多樣自體免疫抗體著稱．Anti-ribosomal P 抗體是其中之一．過去的抗體陽性率調查曾有作者指出海外華人之紅斑狼瘡患者有較高之陽性率，約為 36 至 38%．我們以送來做 anti-dsDNA 檢驗之血清做人類 ribosomal P2 自體免疫抗體的西方墨點檢驗，發現有 38.6%之血清呈陽性反應．我們更進一步去測試 anti-ribosomal P 抗體是否因感染黴菌經由交叉反應而來的假設．因為黴菌或原蟲與 eukaryotic 細胞之 ribosomal P 蛋白類似，部分氨基酸列序幾乎一致．由於 Anti-ribosomal P 抗體之抗原決定部位集中於蛋白之 carboxyl 尾端之 22 個胺基酸，我們以黴菌之 ribosomal P 蛋白非尾端部分為抗原，測試紅斑狼瘡病患是否有對抗黴菌蛋白之抗體．我們從 *P. marneiffei* 及 *C. neoformans* 拿到 P2 之 cDNA，去掉 C 尾端之 13 個胺基酸，以此作為抗原．在全部血清中有 26.9%對其中一種或兩種黴菌抗原有反應．有人類 ribosomal P2 自體免疫抗體的血清也是 28.6%有黴菌蛋白抗體．此一結果雖不支持交叉反應，但亦未完全排除．因對有 P2 自體免疫抗體的病患而言，carboxyl 尾端之 22 個胺基酸有可能是最強的抗原，在抗體成熟的過程中，其他抗體轉弱而消失．

關鍵字：全身性紅斑狼瘡，Anti-ribosomal P 抗體， 黴菌 ribosomal P2 蛋白

英文摘要

The anti-ribosomal P antibodies may have clinical association with psychosis, nephritis, hepatitis and disease activity in patients with systemic lupus erythematosus. It is interesting that oversea Chinese lupus patients have higher prevalence of this antibody than white and black patients. We study the prevalence of anti-ribosomal P antibody in sera sent for detection of anti-dsDNA. Among the 145 sera, the positive rate is 38.6% by western blot. Next, we test the theory that anti-ribosomal P autoantibodies are induced by fungal infection due to cross-reaction because the carboxyl terminal is conserved between fungus and eukaryotic cells. The cDNA and PCR product of fungal ribosomal P2 protein were obtained from *P. marneiffei and C. neoformans* by RT-PCR. The carboxyl terminal 13 amino acids were deleted and PCR product was cloned into expressing vector. 26.9% of the 145 sera have reaction to one or both of the truncated fungal P2 protein. Sera with P2 autoantibodies have similar positive rate of 28.6%. This result does not support the theory of cross-reaction. However, cross-reaction can still be the cause of autoimmunity if C terminus is the major epitope to patients with P2 autoantibodies. Antibodies to other epitopes of low affinity may be lost during the maturation of humoral immunity.

Key words. SLE　Anti-ribosomal P2　Fungal ribosomal proteins

前言

The hallmark of the autoimmune response in patients with systemic lupus erythematosus (SLE) is the presence of antibodies to nuclear antigens. The nuclear autoantigens include native and denatured DNA, histone, the histone-DNA complex and some soluble nuclear RNA proteins, such as *Sm* and *nRNP*. Antibodies to cytoplasmic autoantigens are also detected in patients with SLE. Antibodies to *Ro*, *La* and ribosomal proteins occur in a substantial number of patients [1]. Autoantibodies are helpful in diagnosing SLE or its subsets, but their association with disease activity is often controversial. In clinical practice, the anti-dsDNA titer is regarded as a predictor of disease exacerbation [2]. However, in some patients the persistently high titers are not predictive of flare [3]. Therefore, there is no perfect laboratory monitoring of lupus activity at present.

The diversity of the autoantibodies differentiates SLE from many other autoimmune disorders. Part of this diversity may be explained by the cross-reactivity of anti-DNA antibodies with antigenic epitopes found in a variety of molecules. Anti-DNA antibodies had been shown to react with phospholipids, bacteria polysaccharides, extracellular matrix components and components of cell membrane, cytoplasm or nucleus. Anti-native DNA antibodies can bind to cell surface [4] and result in cellular dysfunction induced by penetration of autoantibodies into living cells [5]. One of the membrane receptors for anti-dsDNA was identified as ribosomal P proteins [6].

文獻探討

The ribosome is formed by a number of structural domains. One of the best characterized domains is the ribosomal stalk, a very flexible lateral protuberance of the large ribosomal subunit of bacteria. In *Escheherichia coli*, the stalk was formed by two dimmers of the acidic proteins L7/L12 (L7 is the N-terminal blocked form of L12), which form a highly stable complex with one molecule of protein L10. The stalk had been shown to play an important role in the function of elongation factors during protein synthesis. In eukaryotic ribosomes, the stalk consists of highly conserved acidic ribosomal proteins. They are called ribosomal P proteins because of being phosphorylated by several protein kinases [18]. On the basis of primary sequence similarities, they are categorized into two groups, P1 and P2. The eukaryotic P1 and P2 proteins form a complex with protein P0 and the 28S rRNA GTPase domain. These P1, P2 proteins show a significant level of conservation among species [19]. Recently, ribosomal P protein had been recognized as a predominant antigen in protozoan infection [20]. Moreover, the B cell epitope of anti- *T. cruzi* ribosomal P is also located in the carboxyl-terminal of the antigen protein, similar to lupus autoantibodies [21]. In addition, fungal ribosomal proteins were identified as fungal allergens [22]. Therefore, eukaryotic ribosomal P proteins can be the targets of human

humoral immunity.

Autoantibodies to ribosomal P proteins was detected and reported in patients with SLE in 1985 [7]. The association between anti-ribosomal P antibodies and psychosis, liver and kidney involvement in SLE had been reported [8-10]. However, other studies failed to show the relationship [11-13]. The frequency of anti-ribosomal P was increased in patients with active disease [12,13] and active nephritis [10]. Furthermore, fluctuations in anti-ribosomal P levels were noted during disease exacerbations and remissions [10,11]. As to the correlation with anti-dsDNA antibodies, 81% of patients with anti-ribosomal P were found to have anti-dsDNA antibodies in one study. Therefore, in addition to the level of anti-dsDNA antibodies, monitor the titer of anti-ribosomal P may be helpful in the longitudinal follow-up of SLE. There is a wide variation in the reported prevalence of the anti-ribosomal P antibodies in patients with SLE. The initial studies showed the frequency varying from 10% to 42% [11,12]. Most reports found a frequency in the range of 10-20%. The high positive rate in Japanese patients (42%) was attributed to the selection of patients with active disease. However, significantly increased prevalence of anti-ribosomal P antibodies (38 and 36% respectively) in Chinese patients was reported in literature [14,15]. The populations studied in both reports are oversea Chinese. In both studies, ELISA was used to detect anti-ribosomal P antibodies. Synthetic peptide P (C terminal 22 amino acid of P0, P1 and P2) or recombinant human P2 was used as coating antigen in ELISA respectively.

研究目的

Recently, we used commercial available anti-P ELISA kit (ImmuLisa anti-ribosomal P, IMMCO Diagnostic New York USA) to detect the prevalence of anti-P autoantibodies in a limited number of patients with SLE. The prevalence was 25% (11/44) in randomly selected patients and 60% (31/52) in patients with high anti-DNA titer (>45 IU). When we reviewed the clinical manifestations of patients with positive anti-ribosomal P antibodies, one patient who had severe interstitial cystitis and bilateral hydronephrosis caught our attention. Additional four patients with documented hydronephrosis who had stored sera collected before and during onset of interstitial cystitis were tested for anti-ribosomal P antibodies. All patients had increased titer of anti-ribosomal P antibodies when hydronephrosis was found. Because the antigen used in commercial kit was not specified in manufacturer's handbook, we decided to make recombinant ribosomal proteins. Human ribosomal gene was cloned by RT-PCR performed on mRNA obtained from human PBMC. The primers were designed according to published sequences [16]. The PCR products of P0, P1 and P2 genes were cloned into TA cloning system and then re-cloned into His-Patch ThioFusion expression vector (Invitrogen California U.S.A). The fusion proteins were purified from transfected *E. Coli* according to manufacturer's suggestion.

We used 200 ng of recombinant proteins to coat each well of polystyrene plate. Our anti-ribosomal P ELISA results were compatible with the commercial kit. The fusion proteins were then transblotted to nitrocellulose membrane for Western blot. But only 2 out of 5 patients with interstitial cystitis had reaction bands to the acidic ribosomal P proteins. It is possible that lupus sera contain antibodies to other component used in ELISA procedure, such as bovine serum albumin [17], or non-specific binding can occur. According to our result, we decide to detect anti-ribosomal P antibodies by Western blot.

Another hypothesis we want to test is that anti-ribosomal P autoantibodies are aroused by fungal or protozoan infection? In the study of *T. cruzi* infection, the antibodies against *T. cruzi* ribosomal P bound less well with human P proteins, but the reactivity detected by ELISA was higher than negative control groups [20]. In this study, we study the prevalence of IgG and IgA anti-ribosomal P antibodies in our patients with SLE. In order to explore the possible role of fungal infection in the development of anti-ribosomal autoantibodies, we clone fungal ribosomal P2 proteins and detect the presence of anti-fungal ribosomal protein in lupus patients with and without anti-human ribosomal P.

研究方法

Methods

Patients

145 sera sent for the detection of anti-dsDNA were tested for anti-ribosomal antibodies without knowing their clinical diagnosis. All sera were collected and stored at −70℃.

Anti- human and fugal ribosomal P2 western blot

The human and fungal ribosomal P2 mRNAs were extracted from human PBMC, *P. marneiffei and C. neoformans.* RT-PCR was done by using the following promers:

Primer for reverse transcriptase: human ATC AAA AAG GCC AAA TCC CAT

Fungus GTC GAA GAG ACC GAA GCC CAT GTC

Primer for PCR: forward: human ATG CGC TAC GTC GCC TCC TAC ATG

Fungus AAG TAC CTC GCA GCA TAC CTC CTC CTC

reverse : human ATC AAA AAG GCC AAA TCC CAT

Fungus GTC GAA GAG ACC GAA GCC CAT GTC

The cDNA was synthesized by Abgene’s first strand synthesis kit (Reverse-iT, Abgene, UK). The cDNA purified by PCR purification kit (QIAquick, Qiagen) was subsequently used in PCR in the following condition: 10 mmol/L Tris HCL, pH 8.3, 50 mmol/L potassium chloride, 2.5 mmol/L magnesium chloride, 200μmol/L of each

dNTP, 1 unit of Taq DNA polymerase (Supertherm, Roche, Germany) and 300 ng of forward and reverse primers. The PCR program is: 94℃ for denaturation of one minute, annealing at 50℃ for 30 seconds and primer extension at 72℃ for 30 seconds for 35 cycles followed by extension for 10 minutes for one cycle. The PCR products were sequenced directly by automatic sequencer and cloned by TA cloning kit. The human ribosomal P2 and the truncated fugal P2 (deletion of the 13 C-terminal amino acids) were cloned into protein-expression vectors. The recombinant P2 proteins were denatured in 1% SDS and 2-mercapto-ethanol by boiling for 10 minutes. After being separated in 15% SDS-polyacrylamide gel electrophoresis, the proteins were blotted to nitrocellulose membranes. The membranes are blocked with 5% skim milk in PBS buffer for 2 hours, washed and dried. The membranes are cut into strips. Each strip will be incubated with test serum 1:100 diluted in skim milk solution overnight at room temperature. After wash with PBS tween-20, peroxidase conjugated goat anti-human IgG is added and incubate at room temperature for 2 hours. The conjugate is washed off and 4-chloro 1-naphthol is used as substrate the visualized the reaction band.

結果與討論

The amino acid sequences of ribosomal P2 proteins cloned from human, *P. marneiffei and C. neoformans* are shown as below, 13 amino acids in the C-terminal are deleted.

1.Cry M K Y L A A Y L L L Q Q G G N A S P S A A D I K A L L E T V G V E A E E
2.Pen G L A G N A S P S A K D I T V V L E S V G I D A D E
3.Hum M R Y V A S Y L L A A L G G N S S P S A K D I K K I L D S V G I E A D D

1.D R L S K L I S E L E G K D I N E V I A E G S S K L A S V P S G G A A P A A A A
2.E R L E K L I S E L E G K D I S E L I A E G S T K L A S V P S G G A G G A P A A
3.D R L N K V I S E L N G K N I E D V I A Q G I G K L A S V P A G G A V A V S A A

1.G G A A A G G A A E A A P A E E K K E E A K
2.G G A A A G G A A A A K E K A E E K E E D K
3.P G S A A P A A G S A P A A E E K K D E K K E E S

40 sera have moderate to strong antibody reaction against human ribosomal P2, another 16 sera react weakly with the P2 protein in the western blots. Only 10 and 6 of the moderate to strong and weak reactive sera respectively contain antibodies against the truncated ribosomal P2 of *P. marneiffei* and/or *C. neoformans*. On the other hand, 23 sera are positive for anti-truncated ribosomal P2 but are negative for anti-human P2.

Our result shows that 27% patients have antibodies against fungal ribosomal P2. The fact that patients with positive human P2 autoantibodies do not have higher frequency of anti-fungal P2 antibodies seems to argue against the theory of cross-reaction between human and fungal antigens. However, cross-reaction is still possible if C-terminal is more efficient in eliciting humoral response or antibodies to epitopes outside the C-terminal gradually faded away during the maturation of humoral immunity.

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