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ANCA 関連腎炎モデル SCG/Kj マウスにおける腎病変と肺血管病変の関連

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【目的】SCG/Kj マウスは血清 MPO-ANCA 陽性で、半月体形成性糸球体腎炎の腎病変について我々は報告してきた。今回は本マウスにおける腎病変と肺病変の関連について検討した。【方法】12～17 週齢雌の SCG/Kj マウスを用い、これらの尿蛋白、血尿、BUN を測定した後、腎臓と肺を摘出して評価した。腎組織は PAS 染色を行い、壊死、半月体形成を評価した。肺組織は HE 染色、EVG 染色、PAM 染色を行い、血管炎の程度を評価した。【結果】腎病変はほとんどの個体でみられ、週齢の進行と共に半月体形成が顕著に増加する個体が多かった。肺病変は約半数にみられ、動脈壁の破壊像とともに周囲に細胞の集簇がみられた。週齢の進んでいないマウスでは腎病変および肺病変のどちらか片方のみが著明であるものもみられた。【結論】SCG/Kj マウスにおける腎病変と肺病変は概ね両者の相関があるものの個々のマウスで個体差が見られる点でヒトの ANCA 関連血管炎の場合と類似しており、本マウスがこの疾患のモデル動物として全身の血管病変を考えるうえでも有用であることが示唆された。

The relationship between renal lesions and lung vascular lesions in SCG/Kj mice as a model of ANCA-associated crescentic glomerlonephritis.

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SCG/Kj mice spontaneously develop crescentic glomerulonephritis (GN) with serum myeloperoxidase anti-neutrophil cytoplasmic antibody (ANCA), as we previously reported. In the present study, we investigated the relationship between renal lesions and lung vascular lesions in SCG/Kj mice. Renal lesions were found in the majority of animals and crescent formation increased with aging. Lung lesions of vasculitis revealed vessel wall destruction and cell proliferation in nearly half of animals. In some young mice, there were severe singly lesions either kidney or lung. Although lung vascular lesions basically correlated with renal lesions, there were some differences among individual mice: some mice presented dominantly renal lesions; and also some other mice, lung vascular lesions in the similar manner of human ANCA-associated microscopic polyangiitis. Present data suggest that SCG/Kj mice are useful as an animal model for this disorder to consider systemic vascular lesions as well as renal lesions.

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ANCA 関連腎炎モデルマウスの発症機序の解析

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ANCA が高値となり、半月体形成性腎炎を来す動物モデルは、ヒト ANCA 関連腎炎(血管炎)の病態解明と治療の確立に最重要課題である。我々は数年前から BSA 誘導腎炎を作製し、ANCA が上昇を認めた。ANCA 上昇は、C57BL/6 や B10 マウスでも認められ、再現性のある実験系であることを確立した。同時に、BSA 誘導による腎炎での ANCA 上昇、半月体形成に至る機序の解析を行った。実験方法は、BSA と complete Feuind's Ajuvant を混合し、2週毎に3回前感作注射する。その後、8週目からBSAを腹腔内に注射し、腎炎を誘発した。その結果、尿蛋白が出現し、腎糸球体への細胞浸潤が起こり 14 週目での屠殺時には半月体形成性腎炎を認めていた。循環末梢血中の血小板や好中球の増加により好中球の活性化をもたらし、ANCA の上昇を出現させる可能性がある。腎糸球体への細胞浸潤は、蛋白尿をもたらす。ANCA や活性化した好中球は血管内皮細胞を障害し、半月体形成性腎炎をもたらすと考えられる。

A novel mouse model for MPO-ANCA-associated glomerulonephritis-Analysis of pathogenesis-

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We demonstrated in BSA induced glomerulonephritis in several mice such as C57BL/6 and B10 mice showed renal damage with deposition of immune complexes. Induction of glomerulonephritis with daily BSA injection after BSA perimmunization showing proteinuria and cells infiltration into glomeruli with changes of platelets, neutrophil and T and B cells in circulating peripheral blood. Crescentic formation in kidney was observed at 14 weeks, but glomerular change did not expressed during BSA preimmunization. Peripheral platelets and neutrophils significantly increased in contrast to decrease of decrease of T and B cells. Proteinuria with increasae of TNF was significantly increased at 11weeks after daily BSA injection, the frequency of hematuria recognized by 11-50%.. Between MPO-ANCA and neutrophil infiltratin into glomeruli related, and high titer of MPO-ANCA related significantly proteinuria. Proteinuria markedly related to neutrophil infiltration into glomeruli. Especially, crescentic glomeruronephritis may be induced neutrophil infiltration into glomeruli with increase of ANCA in peripheral blood. Theses observation suggest that MPO-positive activated neutrophil infiltration against glomerullar capillary walls directly may be involved subendotherial cells, and then may be expressed creacentic glomerulonephritis with hematuria.

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Effect of immune complexes in serum from patients with rheumatoid vasculitis on the expression of cell adhesion molecules on polymorphonuclear cells.

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OBJECTIVE: Immune complexes (IC) are frequently detected in patients with rheumatoid vasculitis (RV). To explore the pathogenic role of IC in the development of vasculitis among patients with rheumatoid arthritis (RA), we examined the effect of IC on the expression of cell adhesion molecules (CAM) on polymorphonuclear cells (PMN). **METHODS:** PMN from healthy volunteers were incubated with the sera from 26 patients with RA including 9 patients with RV, and the expression of CAM on the PMN was assessed by flow cytometry. **RESULTS:** We found that 67% (6/9) of the serum samples from RV patients and 18% (3/17) of the samples from RA patients without RV revealed up-regulated CD11b expression. On the other hand, 89% (8/9) of the samples from RV patients and 12% (2/17) of the samples from RA patients without RV revealed up-regulated CD18 expression. However, the expression of CD11a was not affected. Up-regulation of CD11b and CD18 on PMN was also induced by the immunoglobulin G (IgG) fraction of the sera of RV patients. Moreover, L-selectin expression on PMN was down-regulated by the sera or IgG of some patients with RV. These changes in CAM expression on PMN induced by IgG of RV patients were not observed when PMN were incubated with the IgG of RV patients from which the IC formed by IgG had been removed. **CONCLUSION:** These results suggest that IC formed by IgG in patients with RA are involved in the development of vasculitis by affecting the expression of CAM on PMN (Clin Exp Rheumatol. 2001;19(1):59-68.)

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Usage of Quantum dots for the evaluation of vasculitis

我々は、定量ドット(QD)標識抗 MPO を作成した。これを用いて自然発症型糸球体腎炎モデルマウスならびに MPO-ANCA 関連糸球体腎炎の患者の好中球における myeloperoxidase(MPO)の役割を検討した。QD 標識抗 MPO 抗体を用いることで、健常人の好中球は炎症性サイトカインである IL-1,IL-8 ならびに TNF-alpha 刺激によりその表面上に MPO を表出させることが判明した。またマウス好中球においても同様の活性化が観察された。その一方で、腎糸球体腎炎モデルマウス(SCG/Kj)では、未刺激好中球においてもその表面に MPO の表出が観察された。さらに、ANCA 関連糸球体腎炎を発症した一部患者において、モデルマウスと同様に未刺激状態でも好中球表面へ MPO が表出していることが判明した。これらの結果は、抗 MPO Ab をにより検出可能である活性化好中球上に表出された MPO が、糸球体腎炎の発症および進行の第一歩に重要な役割を果たす可能性があることが示唆された

Usage of Quantum dots for the evaluation of vasculitis

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We examined the role of myeloperoxidase (MPO) and the Ab to MPO in the pathogenesis of glomerulonephritis associated with MPO-specific anti-neutrophil cytoplasmic auto-Ab (MPO-ANCA) in experimental glomerulonephritis mice using quantum dots (QDs). We demonstrated the QD-conjugated anti-MPO Ab visualized the expression of MPO on the neutrophil surface after stimulation with proinflammatory cytokines. It is notably that the spontaneous crescentic glomerulonephritis model mouse (SCG/Kj mice) showed the expression of MPO on the neutrophil surface even in resident state. Moreover, We also observed that MPO translocation to the surface of neutrophils in patients with rapid progressive glomerulonephritis without any stimulation, suggesting that MPO translocation may be certain to contribute to the development of glomerular lesion.

These results indicate that the expressed MPO on the activated neutrophils with anti-MPO Ab may coordinately play essential roles in the initial steps for the development of glomerulonephritis.

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血管炎の診断および治療におけるnMPO-ANCAの有用性
—MPO-ANCAとnMPO-ANCAの解離について—

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目的と方法
nMPO-ANCA は健常人の好中球より抽出したミエロペルオキシダーゼ (MPO) に対する抗体であるが, 日常臨床において検査会社に依頼する commercial base のキットを用いた MPO-ANCA の測定結果と一致しない症例が散見される。広島市民病院で 2004 年 6 月～2005 年 9 月の間に ANCA 陽性の症例を 12 例経験したが, nMPO-ANCA の臨床的有用性を検討するため, nMPO-ANCA と MPO-ANCA を同時に測定し, 症例を解析した。
結果 [nMPO ＋, MPO －] 5 例(うち 1 例は nMPO ＋, MPO ±), [nMPO －, MPO ＋] 2 例(うち 1 例は測定日に 5 日のずれがあるため厳密には解離疑い), [nMPO ＋, MPO ＋] 5 例
[nMPO ＋, MPO －]の 5 症例はいずれも顕微鏡的多発血管炎(MPA)診断基準の確実例としての基準は満たさなかったが, 発熱, 炎症反応上昇等の臨床症状および検査所見より細小レベルでの血管炎の存在が強く疑われた。これらのうち経時的に測定し得た 3 症例では疾患活動性の低下に伴って nMPO-ANCA は陰性化した。
[nMPO －, MPO ＋]の 1 症例は胸鎖関節炎に間質性肺炎を合併した症例であるが, MPO-ANCA 値と, CRP 値や間質性肺炎の活動性を示す KL-6 値は相関せず, 本症例における MPO-ANCA 値は疾患活動性を反映しないと考えられた。
まとめ
現在までの少数例の検討では nMPO-ANCA の方が MPO-ANCA よりも血管炎に対する感度が高く, 定型的な MPA 以外の血管炎を拾い上げる可能性が示唆された。 nMPO-ANCA の臨床的有用性を明確にするため, さらなる症例の蓄積を行い病態を解析する必要がある。

Usefulness of nMPO-ANCA in diagnosing and treating vasculitis
-discrepancy between MPO-ANCA and nMPO-ANCA-

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Background nMPO-ANCA is anti-neutrophil cytoplasmic antibodies (ANCA) targeting to human neutrophil-derived myeloperoxidase (MPO) from healthy control subjects. Recent analysis has revealed some discrepancy of titer between nMPO-ANCA and commercial-based MPO-ANCA, which is utilized in almost all general practice.
Purpose To analyze clinical characteristics of patients with nMPO-ANCA, and determine usefulness of nMPO-ANCA in diagnosing and treating patients with vasculitis.
Results
12 cases showed ANCA positive between June 2004 and September 2005 in Hiroshima City Hospital.
[nMPO-ANCA positive / MPO-ANCA negative]: 5 cases
[nMPO-ANCA negative / MPO-ANCA positive]: 2 cases (one is suspected case)
[nMPO-ANCA positive / MPO-ANCA positive]: 5 cases
All 5 cases with [nMPO-ANCA positive / MPO-ANCA negative] did not satisfy Japanese criteria of microscopic polyangiitis (MPA) as definite cases, but were strongly suspected of having vasculitis involving small vessels, considering clinical symptoms and laboratory data. nMPO-ANCA was measured serially in 3 of 5 cases. In these 3 cases, positive nMPO-ANCA turned to be negative, accompanied by decrease of disease activity.
One case with [nMPO-ANCA negative / MPO-ANCA positive] suffered from arthritis of sternoclavicular joint and interstitial pneumonia (IP), and the levels of MPO-ANCA did not correlate with those of KL-6, a serum marker of IP, nor CRP. Therefore, MPO-ANCA in this case did not reflect disease activities of IP and arthritis.
Conclusion The present data in a small number of cases suggested that nMPO-ANCA could be more sensitive against vasculitis compared to commercial-based MPO-ANCA, and could detect atypical case of MPA. Further accumulation of cases is necessary for clarifying usefulness of nMPO-ANCA in general practice.

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紫外線誘発皮膚炎における好中球由来活性酸素の関与

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活性化した好中球は、NADPH オキシダーゼによって酸素からスーパーオキシドを、ミエロペルオキシダーゼ(MPO)によって過酸化水素から次亜塩素酸を産生する。紫外線(UVB)が皮膚に照射されると、皮膚組織への好中球の浸潤が起こる。そのため、UVB 誘発皮膚炎において、好中球から産生される活性酸素が皮膚組織に傷害を与えている可能性が高いが、詳細は明らかでない。本研究では、MPO ノックアウトマウス(MPO-KO マウス)、NADPH オキシダーゼ欠損マウス(CGD マウス)、およびその両酵素の二重欠損マウス(MPO-KO/CGD マウス)を用いて、紫外線誘発皮膚炎における好中球由来の活性酸素の関与を個体レベルで解析した。UVB をマウスの背部に照射し、実験的皮膚炎を誘発させ皮膚の組織病理像を比較したところ、野生型マウスよりも MPO-KO マウスの方が早期に皮膚炎が進行し、CGD マウスや MPO-KO/CGD マウスはさらに早期に進行した。また、炎症細胞の多くが好中球であることを同定した。変異マウス好中球はケモカイン走化性が野生型好中球よりも亢進していることが、各ノックアウトマウスの方が炎症が早期に進行する一因であることが示唆された。

Role of Neutrophil-derived ROS for the Ultraviolet-induced Skin Inflammation

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Exposure of the skin to UVB causes acute inflammation. Myeloperoxidase (MPO) and NADPH-oxidase are located mainly in neutrophils, which catalyze the reaction to produce hypochlorous acid and superoxide, respectively. Mice genetically deficient in either MPO (MPO-KO mice) or NADPH-oxidase (CGD mice), or in both enzymes (MPO-KO/CGD mice), and wild-type mice were exposed to UVB to induce inflammation. Interestingly, all of the mutant mice showed more rapid onset of UVB-induced inflammation than the wild-type mice. Many of the infiltrating cells were histochemically identified as neutrophils. Neutrophil migration toward KC was higher in mutant than wild-type mice. These results suggest that ROS produced by neutrophils regulate migration of neutrophils toward KC. This may explain the earlier infiltration of mutant neutrophils in response to UVB.

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人工ポリクローナル免疫グロブリン

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免疫グロブリン大量静注療法(IVIG)は ANCA(anti-neutrophil cytoplasmic antibody)が関与する川崎病、血管炎、腎炎等の治療に有効である。しかしながら、その安全性、副作用や安定供給面での問題を抱えており、人工グロブリンの開発は極めて重要である。
我々は、大腸菌のシステムで人工ポリクローナル Fv (poly-Fv; マウス脾臓由来)及びヒト Fc ドメイン(CH1-CH2-CH3)を生産する技術を構築した。現在、poly-Fv、poly-Fv 及び Fc のミクスチャー、あるいは Fc 単独のCAWS誘発血管炎疾患モデルマウスにおける治療効果を検証中である。また、50 人の健常者から得た末梢血 RNA より poly-Fv も作製中である。

Synthetic polyclonal immunoglobulin

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While IVIG (intravenous immunoglobulin) treatment is effective for ANCA (anti-neutrophil cytoplasmic antibody)-associated Kawasaki disease, vasculitis, and glomerulonephritis, immunoglobulin from plasma has problems in safety, side effects, and stable supply. Development of synthetic polyclonal immunoglobulin is pressing need.
We developed production methods of *E. coli*-derived polyclonal Fv (poly-Fv) from mouse spleen antibody genes and human antibody Fc domain (Fc; CH1-CH2-CH3). Now we try therapy of CAWS-induced model mice of human vasculitis with poly-Fv, mixture of poly-Fv and Fc, or Fc. Synthetic human poly-Fv is also under preparation from peripheral blood RNA of 50 persons in normal health.

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血管炎患者の血液細胞で特異的に発現亢進している遺伝子群の包括的単離

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我々が独自に開発した段階的サブトラクション(重差分)法により、さらにDNAチップ(アジラント社Hu44K)を補完的に用いて、血管炎の末梢血液細胞で健常人に比べて特異的に転写誘導されている遺伝子の包括的な単離を試み、100種類遺伝子の遺伝子を獲得した。対象とした血管炎は、顕微鏡的多発血管炎(MPA)、高安動脈炎(TA)、ウエゲナー肉芽腫症 (WG)、抗リン脂質抗体症候群(APS)、アレルギー性肉芽腫血管炎(AGA) 、悪性関節リウマチ(MRA)、側頭動脈炎(GCA)、バージャー病(BD)、結節性多発動脈炎(PN)である。まず、これらの遺伝子のいくつかについて、個々の患者の血液細胞を対照としてリアルタイムPCRにより患者ごとの発現量を比較したところ、多くの患者で共通に転写誘導されている遺伝子が見つかった。これらが自己免疫疾患の患者の末梢血液細胞でも共通に転写誘導されているかどうかも調べるため、慢性関節リウマチ(RA)、全身性エリテマトーデス(SLE)、自己免疫性血小板減少性紫斑病(ITP)という他の3つの患者の血液細胞での発現量もリアルタイムPCRで検索した。なお、免疫不全疾患ではあるが自己免疫疾患ではない重症アトピー患者を対照として用いた。これらのアプローチ法は自己免疫疾患共通の原因遺伝子の同定に役立つと信じている。

Comprehensive isolation of the genes that are specifically expressed in the blood cells of angiitis patients

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We have comprehensively isolated the disease specific mRNA (cDNA) species whose expressions are extraordinarily augmented in blood cells of angiitis patients as compared to those of normal volunteers. These patients are affected by either Microscopic Polyangiitis (MPA), Takayasu's Arteritis (TA), Wegener's Granulomatosis (WG), Antiphospholipid Syndrome (APS), Allergic Granulomatous Angiitis (AGA), Malignant Rheumatoid Arthritis (MRA), Giant Cell Arteritis (GCA), Buerger's Disease (BD) or Polyarteritis Nodosa (PN). Real time PCR revealed that expression levels of some of these genes are largely enhanced in the blood cells of most of the patients. To identify the common genes whose expressions are augmented in many of the autoimmune diseases, we also compared their expressions in the patients affected by other autoimmune diseases such as Rheumatoid Arthritis (RA), Systemic Lupus Erythematodes (SLE) or Idiopathic Thrombocytopenia Purpura (ITP). Patients affected by atopy were also examined as a control of non-autoimmune disease. We believe that this approach may help to identify the autoimmune specific genes.

研究成果の刊行に関する一覧

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A Novel Mouse Model for MPO-ANCA-Associated Glomerulonephritis

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Abstract: **We established a novel model mouse for myeloperoxidase anti-neutrophil cytoplasmic antibody (MPO-ANCA)-associated glomerulonephritis with crescentic formation, which was induced by administering bovine serum albumin (BSA). Neutrophil infiltration into the renal glomeruli began at 8 weeks and crescent formation was observed from 10 weeks after the first BSA injection. Platelet and neutrophil counts significantly increased, and proteinuria was observed from 5 weeks. MPO-ANCA increased slightly at 4 and markedly at 9 weeks, and the TNF-α level increased at 11 weeks. Glomerular neutrophil infiltration was correlated with MPO-ANCA levels. In addition, proteinuria also significantly correlated with MPO-ANCA levels. Finally, renal crescent formation was associated with an increase of MPO-ANCA levels and neutrophil infiltration into glomeruli. The glomerular immune deposition of IgG and C3 was observed. These findings indicate that BSA induces neutrophil activation of peripheral blood followed by the elevation of MPO-ANCA, resulting in the development of crescentic glomerulonephritis in mice.**

Key words: **MPO-ANCA, BSA-induced crescentic glomerulonephritis, Activated neutrophils, ANCA-associated glomerulonephritis**

It has been shown that myeloperoxidase (MPO) and the MPO-specific anti-neutrophil cytoplasmic auto-antibody (MPO-ANCA) are risk factors for the development of glomerulonephritis (GN). High titers of MPO-ANCA are frequently detected (2, 4, 5, 8) in the sera of patients with microscopic polyangiitis (MPA) or crescentic GN (CrGN). Animal models have been used for understanding the mechanisms for the development of vasculitis, as a basis for establishing new therapeutic strategies. The SCG/Kj mouse is a model of spontaneous CrGN associated with activated neutrophils (6). The MRL lpr/lpr strain, the background for the SCG/Kj mouse, is known to show high levels of MPO-ANCA in association with renal lesions, including GN and vasculitis (3). MPO-deficient model mice for coronary arteritis induced with *Candida albicans*-derived substances indicate that MPO is a major antigen for MPO-ANCA production (7). The importance of MPO-ANCA for the development of vasculitis has been demonstrated, using immune-deficient mice with the Rag2 knockout (19). Such a mouse model for renal-damage CrGN with MPO-ANCA production is needed, since understanding the pathogenetic roles of MPO-ANCA and neutrophils in GN and vasculitis remains important for the development of new therapies. Bovine serum albumin (BSA)-induced nephritis was reported in rat (12). Recently, CrGN has been connected with MPO-ANCA production prior to the development of renal lesion. We were interested in knowing whether the development of CrGN induced by BSA administration is related to an increase in MPO-ANCA.

In the present study, we established induction model mice for CrGN with vasculitis by serial injection of BSA. The mice showed three phases of renal damage:

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Abbreviations: ANCA, anti-neutrophil cytoplasmic auto-antibody; AP, alkaline phosphatase; BSA, bovine serum albumin; BUN, blood urea nitrogen; Cr, crescentic; FITC, fluorescein isothiocyanate; GN, glomerulonephritis; PE, phycoerythrin; IC, immune complex; MPA, microscopic polyangiitis; MPO, myeloperoxidase.

The initial phase: an increase of peripheral neutrophil and platelet counts and a slight increase in MPO-ANCA, *the second phase*: renal lesions with proteinuria and glomerular neutrophil infiltration, and *the final phase*: a high titer of MPO-ANCA and CrGN. This induction model may explain the sequence of clinical stages leading to renal failure in MPO-ANCA vasculitis in humans.

Materials and Methods

Reagents. BSA and phosphatase substrate were purchased from Sigma Chemical Co. (Mo., U.S.A.). Alkaline phosphatase (AP)-labeled anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (Pa., U.S.A.), AP-labeled anti-rabbit IgG antibody was purchased from Bio-Rad Corp. (Calif., U.S.A.). A kit for the mouse TNF-α immunoassay was purchased from BD Biosciences Pharmingen (Calif., U.S.A.). Urine biochemical assay sticks were purchased from Bayel Medical Corp. (Tokyo).

Animals. Six-week-old female C57BL/6N mice were purchased from Charles River Breeding Laboratories (Shiga, Japan), for comparison of our study with other vasculitis studies (7). Because we initially established MPO-deficient mice for the determination of MPO involvement in MPO-ANCA vasculitis, we used C57BL/6 mice for this study. In addition, this strain has already been used for the study of vasculitis (7). Ten mice in each group were used in the initial step. The mice were housed at constant room temperature (21–26 C) with a 12-hr light/dark cycle under specific pathogen-free conditions in groups of 5 to 6 animals per cage and were allowed standard laboratory chow (CRF-1, Oriental Yeast, Chiba, Japan) and water *ad libitum*. One week later, 7-week-old mice with no abnormalities, in generally good condition, and with no weight gain were selected and used. Animal care methods and experimental protocols for the experiment were based on the Guidelines for Animal Experiments from Nihon Pharmaceutical, Izumisano, Japan.

Experimental design for BSA injection and blood analyses. GN was induced by preimmunization by 8 weeks of subcutaneous injections of BSA (endotoxin free, Sigma A8806), 0.2 mg/mouse in complete Freund's adjuvant at 2-week intervals and subsequent daily intraperitoneal injection of 50 mg/kg BSA for 6 weeks, for a total 14 weeks. Randomly selected mice were sacrificed at 4, 8, 9, 10, 11, 12, 13 and 14 weeks after the first BSA injection. For a control, complete Freund's adjuvant without BSA was injected at 2-week intervals. Blood samples were drawn from the abdominal aorta, by centrifugation, and this plasma sample was used for blood measurement. Kidneys and lungs were fixed with 10% formalin neutralized with phosphate-buffered saline for observation by light microscopy and immunohistochemistry.

Blood cell counts, blood urea nitrogen (BUN), creatinine levels, proteinuria, and hematuria. White blood cell and platelet counts were determined using a multi-automatic blood cell counter (K-2000, Sysmex, Kobe, Japan). Neutrophil counts were determined with Wright-Giemsa staining of blood smears. BUN and creatinine levels in plasma were determined by the enzyme and Jaffe methods, respectively. Proteinuria and hematuria were determined every week after the first injection of BSA by using a clinical stick as a marker for the onset and development of GN. The gradation of proteinuria was determined by hematostick according to the following scores: 0, negative; 1, below 30 mg/dl; 2, 30–100; 3, 100–300; and 4, over 300.

Profiles of leukocytes of peripheral blood by flow cytometry. Peripheral blood cells were collected and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 antibodies, phycoerythrin (PE)-conjugated anti-mouse CD19 antibodies, biotin-conjugated anti-mouse Gr-1 antibodies, and allophycocyanin-conjugated anti-mouse CD11b antibodies, from BD Biosciences (Calif., U.S.A). After incubation with biotin-conjugated anti-mouse Gr-1 antibodies, the cells were subsequently incubated with streptavidin-conjugated phycoerythrin-Cyanine 7 (BD Biosciences). Flow cytometric analyses were performed with a *Cytomics™ FC 500* (Beckman Coulter, Tokyo).

Measurement of mouse MPO (mMPO)-ANCA titer and TNF-α in plasma. Plasma was prepared from heparinized blood derived from the mouse abdominal aorta. Two micrograms per milliliter of recombinant mMPO (rmMPO), prepared as described elsewhere (6), was coated onto an ELISA plate (TS Plate, Toyoshima Co., Tokyo). Subsequently, 15 mM sodium carbonate and 35 mM sodium bicarbonate buffer (pH 9.6) were added to the plate overnight at 4 C. The plate was washed three times with washing buffer (0.005% Tween-20 in PBS without calcium and magnesium) and blocked with 100 μl of PBS containing 1% BSA for 2 hr at room temperature. The plate was then washed again, and diluted mouse plasma was added for 1.5 hr at room temperature. The plate was washed and subsequently the AP-labeled anti-mouse IgG antibody (×4,000 dilution) or AP-labeled anti-rabbit IgG antibody (×3,000 dilution) was added and left to sit for 2 hr at room temperature. After the plate was washed again, *p*-nitrophenylphosphate as an AP substrate was added at a concentration of 1 mg/ml dissolved in substrate buffer consisting of 50 mM sodium carbonate and 1 mM mag-

nesium di-chloride (pH 9.6). After incubation for 60 min at room temperature, absorbance at 405 nm was measured by the Automatic Analyzer (BioRad Corp.). The titer of anti-rmMPO antibody in mouse plasma was determined as equivalent rabbit anti-rmMPO antibody (μg/ml), which was used as a standard. TNF-α concentration in mouse plasma was measured with an ELISA kit (BD Biosciences Pharmingen).

Histological observations of kidney and lung damage. Kidneys and lungs were preserved in phosphate-buffered 10% formalin (Wako, Tokyo), after which the kidneys were chopped into small pieces, embedded in paraffin wax, cut into 3-μm sections, and stained with hematoxylin-eosin and periodic acid Schiff stain. The excised kidneys were processed for light microscopic observation, according to standard procedures. Histological changes in neutrophil infiltration and crescent formation in 50 glomeruli were analyzed (all sacrificed, N=7–10). Neutrophil infiltration was graded according to the following scores: 0, no change (not detected); 1, mild (less than three neutrophils into the glomeruli); 2, moderate (3–15 neutrophils into the glomeruli); and 3, marked (more than 15 neutrophils into the glomeruli). Crescent formation was defined as the presence of at least two or more epithelial cell layers within the Bowman's space. Evaluations were made by a blinded observer.

Determination of neutrophils in kidney using immunohistological staining with myeloperoxidase antibody. Neutrophils were confirmed by an indirect method with a polyclonal rabbit antibody against rmMPO, as described previously (6). Briefly, paraffin sections were incubated with the primary antibody followed by goat anti-rabbit immunoglobulins conjugated to peroxidase labeled-dextran polymer (EnVision^{+TM}, DAKO Corp., Calif., U.S.A). The color was then developed by incubation with a Liquid DAB Substrate-Chromogen System (DAKO Corp.).

Renal immunohistochemistry. Excised kidneys were embedded in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo) and snap-frozen. On a cryostat, 4-μm sections were stained with anti-mouse IgG (ICN, Pharmaceuticals, Inc., Cappel, Calif., U.S.A.), anti-mouse IgM (Biomedica Co., Calif., U.S.A.), anti-mouse IgA (Serotec Ltd., Oxford, U.K.), and anti-mouse C3 (ICN Pharmaceuticals, Inc.). Immunohistochemistry findings were visualized according to the localization of deposits in the glomeruli.

Statistical analysis. Data values were expressed as means±S.D. All statistical differences between groups were determined by Student's t test, and differences among multiple groups were performed by one-way analysis of variance followed by Fisher's least-significant differences method using the SAS program (Version 8.02; SAS Institute, Cary, N.C., U.S.A.). A P value of less than 0.05 was considered statistically significant.

Results

Light Microscopy of Kidney and Lung after Injection of BSA

We observed histological development from proliferative GN to CrGN with glomerular neutrophil infiltration at 8 weeks after the first BSA injection. Crescent formation began at 10 weeks, affecting 4–44% of the 50 glomeruli observed. All animals had several renal histological changes, i.e., crescentic formation and proliferative changes. Glomerular changes were not seen before daily BSA injection at 4 weeks (Fig. 1-A), occurred 8 weeks after the first injection, and developed into severe CrGN at 14 weeks (Fig. 1-B). On the other hand, histological changes in the lungs, such as interstitial pneumonia, were marked 4 weeks after BSA injection (Fig. 1-C). These histological changes in the lungs were also seen in final observations at 14 weeks (Fig. 1-D). However, no histological changes were seen in kidneys and lungs of control mice administered the adjuvant only.

Comparison of Leukocyte Populations in Peripheral Blood after Injection of BSA with Flow Cytometry

After the end of the initial administration of BSA, peripheral leukocytes and platelet counts continued to increase (data not shown). Because of this increase in leukocytes, we compared leukocyte counts by flow cytometry 4 and 14 weeks after BSA injection. Then, Gr-1/CD11b double-positive cells, but not CD3- and CD19-positive lymphocytes (Fig. 2), showed significantly higher counts at 14 than at 4 weeks.

Evaluation of Urinary Protein and Percentage of Hematuria after BSA Injection in Mice

Proteinuria appeared in some mice 5 weeks after the first injection of BSA compared with control (adjuvant only). Proteinuria continued to increase after 5 weeks (data not shown), and 8 weeks after BSA injection, the number of mice with proteinuria and the levels of proteinuria showed a significant increase (Table 1). The appearance of proteinuria indicated an early stage of GN after the injection of BSA, but hematuria appeared later, with the frequency of hematuria rising 11–50% from 11 and 14 weeks after injection of BSA (Table 1). Some mice showed high levels of BUN with no increase of body weight, but this was not seen in all mice with high-titer MPO-ANCA or proteinuria and

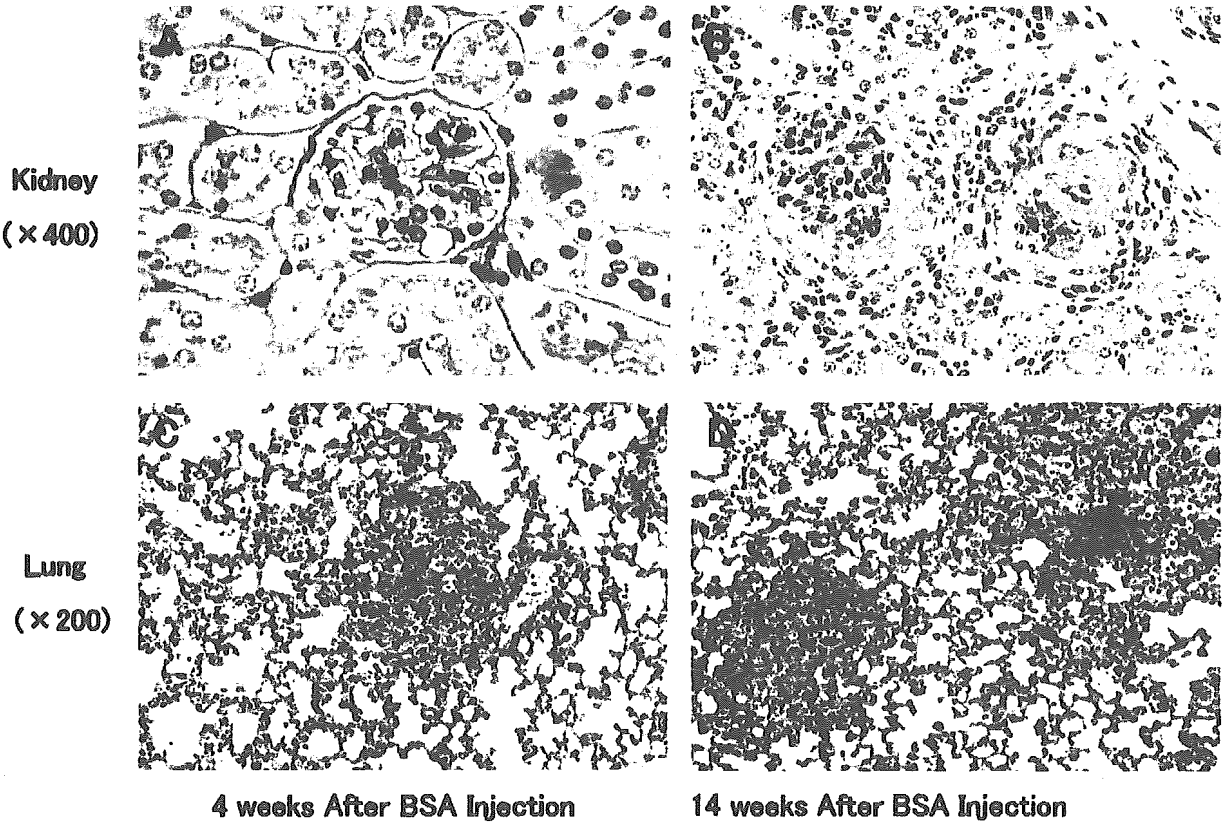

Fig. 1. Morphological change of kidney and lung induced with BSA. Kidney at 4 (A) and 14 weeks (B) after injection of BSA. Periodic acid-Schiff stain (×400). Lung at 4 (C) and 14 weeks (D) after injection of BSA. Hematoxylin-eosin stain (×200).

was not associated with marked elevation of serum creatinine. In addition, gastrointestinal bleeding was not observed.

Increase of Levels of MPO-ANCA and TNF-α after BSA Injection

C57BL/6N mice do not express MPO-ANCA constitutively. However, immediately after the daily injection of BSA at 9 weeks, MPO-ANCA increased markedly, followed by a slight increase at 4 weeks (Fig. 3-A and -B). MPO-ANCA in the sera was not shown to be crossreactive to BSA. TNF-α also increased 11 weeks after BSA injection (Fig. 3-C); in addition, anti-dsDNA antibody did not increase at any time during this experiment (data not shown).

Neutrophil Infiltration into Glomerulus and Correlations among MPO-ANCA Titer, Neutrophil Infiltration, and Proteinuria

Glomerular neutrophil infiltration was observed continuously from 8 weeks (Fig. 4-A), and the cells were confirmed as neutrophils with MPO antibody (Fig. 4-B). Hence, the correlation between MPO-ANCA and renal damage was examined, because these parameters were increased during the administration of BSA into the mice. Neutrophil infiltration was significantly correlated with a high titer of MPO-ANCA (Fig. 4-C). Proteinuria was also significantly correlated with MPO-ANCA titer (Fig. 4-D) and infiltration of neutrophils (Fig. 4-E). At 8 weeks after injection of BSA, when proteinuria was present, neutrophil infiltration in some glomeruli was seen. A high incidence of mice had glomerular neutrophil infiltration 1 week after daily BSA injection. Massive proteinuria was closely related to neutrophil infiltration into the glomeruli (Fig. 4-E).

Immunohistochemistry of IgG and C3 after Injection of BSA into Kidney

Significant glomerular immune deposits were present both in the mesangium and along the capillary wall in proliferative GN after injection of BSA. Proliferative changes in GN were associated with the immune deposition of IgG, IgM, and C3 but not of IgA (data not shown). Finally, mice injected with BSA showed the development of crescentic change during the experiment. However, a high titer of MPO-ANCA was not

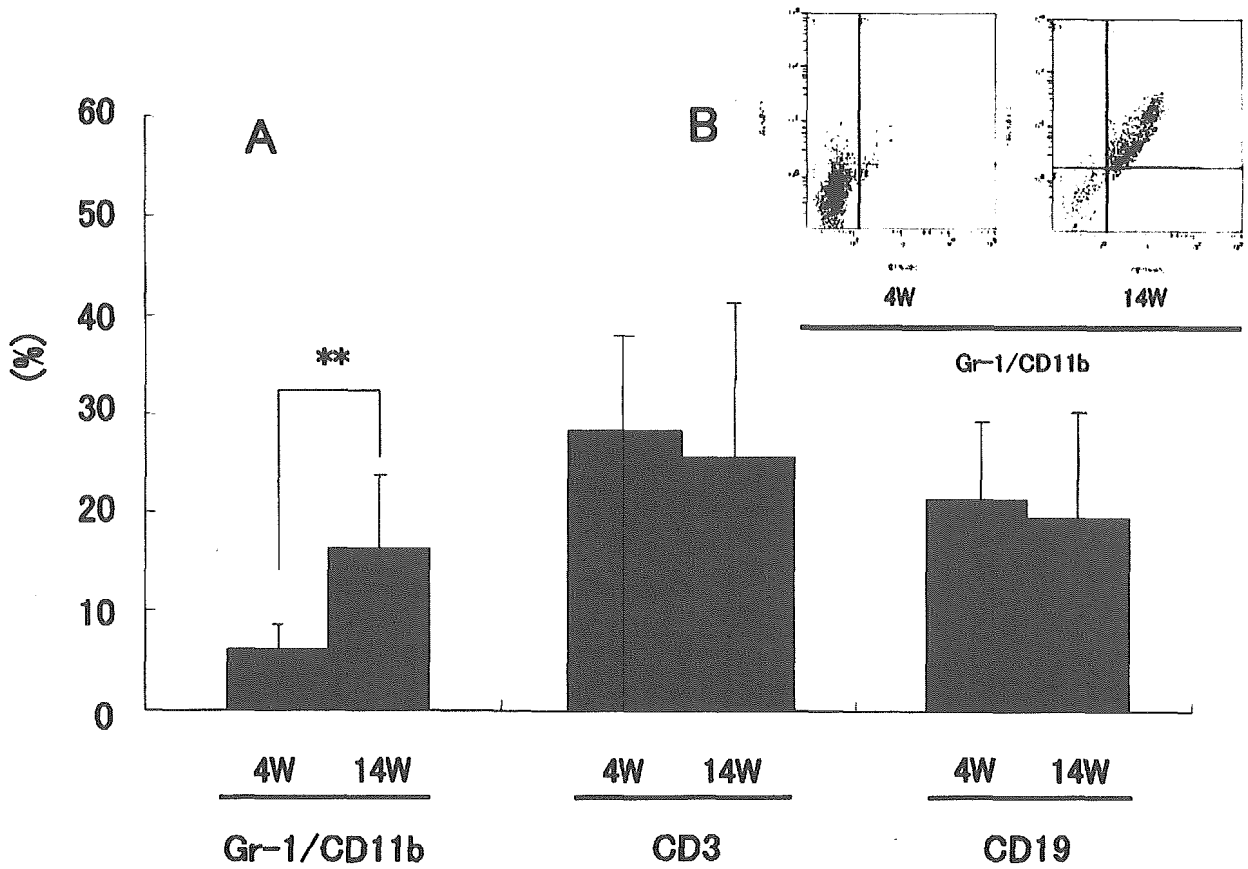

Fig. 2. Comparison of leukocyte populations in peripheral blood after injection of BSA with flow cytometry. (A) Populations of Gr-1/CD11b, CD3, and CD19 in peripheral blood between 4 and 14 weeks were compared. Mean±S.D., $^{**}P<0.01$, compared between 4 ($N=10$) and 14 weeks ($N=7$). (B) Profiles of Gr-1/CD11b population at 4 and 14 weeks.

Table 1. Evaluation of urinary protein and percentage of hematuria after BSA injection in mice

Group	Weeks after BSA injection			
	0	8	11	14
	Proteinuria (mg/dl)			
BSA injected	21±14	58±35*	132±107*[a]	414±410*[b]
Control (adjuvant only)	16±16	20±14	26±10	23±12
	Urine positive for hematuria (%)			
BSA injected	0	0	13	50
Control (adjuvant only)	0	0	0	0

Mean±S.D. $^{*}P<0.01$, compared with control (adjuvant only).

$N=10$ except for group of BSA (a) and (b). (a) $N=8$, decease: 1 mouse at 9 weeks (shock), 1 mouse at 10 weeks (renal failure) from the initial $N=10$. (b) $N=7$, decease: 1 mouse at 9 weeks (shock), 2 mice at 10 weeks (renal failure) from the initial $N=10$.

related to the percentage of crescent formation. On the other hand, histological damage occurred in the lungs early after BSA injection, before MPO-ANCA elevation or the appearance of massive proteinuria.

Discussion

The induction of CrGN, together with variable changes in other organs following BSA injection, has