J Tokyo Wom Med Univ
71 (7・8) 377～386 (2001)

Effect of Soluble Form CTLA-4 on Spontaneous IgA Nephropathy in ddY Mice

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(Received Mar. 19, 2001)

The aim of the present study was to examine the role of CD28-B7 signaling in the development of glomerulonephritis in ddY mice, an animal model for IgA nephropathy (IgAN). To achieve this aim, we investigated whether or not the CTLA-4 (CD152) fusion protein, which binds to B7.1 (CD80) and B7.2 (CD86), affects glomerular pathological changes, including IgA deposition, or functional parameters, such as serum creatinine and proteinuria. Each group (n=4) was given either human CTLA-4 fused with human IgG (CTLA-4Ig) or control human IgG_1. All treated groups of mice were injected intraperitoneally at a dose of 0.1 mg twice a week for the duration of the study. Mice given control human IgG_1 progressively developed typical mesangioproliferative glomerulonephritis, with remarkable glomerular IgA deposits. In contrast, mice treated with CTLA-4Ig showed a significant reduction in proteinuria and mesangioproliferative change, with an expansion of the mesangial matrix at 40 weeks of age. The serum IgA levels of these mice were considerably lower than those in mice given the control human IgG_1. A direct immunofluorescence study showed the reduction of glomerular IgA deposits in CTLA-4Ig-treated mice. We have demonstrated for the first time that the development of spontaneously occurring IgA nephropathy can be prevented in ddY mice by blocking the CD28-B7 interaction using a soluble form of CTLA-4Ig. These results suggest that a costimulatory signal via CD28-B7 may play a crucial role in the development and progression of IgAN.

Introduction

The first interaction between T cells and professional antigen presenting cells (APC) is regulated by two signals. The first signal is provided by the binding of a peptide: major histocompatibility complex (MHC) with a T-cell receptor (TCR) and represents an antigen-specific response. However, this ligation does not stimulate naive T cells to proliferate and differentiate into effector T cells. The activation of naive T cells requires a second signal, the costimulatory signal, delivered by the same APC. The best-characterized costimulatory signal for an interaction between T cells and APC is provided by the binding of CD28 and CTLA-4 (CD152) on T cells to B7.1 (CD80) and B7.2 (CD86) on APC[1)~4)]. CTLA-4 is a homologue of CD28, which also binds to B7 molecules on APC and delivers a negative signal

for T cell stimulation, such as the down-regulation of interleukin 2 (IL-2) production, IL-2 receptor expression, and cell cycle progression in vitro[5)~8)]. Because CTLA-4 binds B7 molecules about 20 times more avidly than CD28, a soluble form of CTLA-4 has been used to inhibit T cell costimulation via CD28. Blockade of this pathway has been shown to inhibit autoimmune responses in vivo[9)~12)].

IgA nephropathy (IgAN) was originally described by Berger[13)], and primary IgAN, characterized by the accumulation of IgA deposits in the glomerular mesangium, is the commonest form of glomerulonephritis in the world [14)]. About half of all renal biopsies performed between 1985 and 1993 were diagnosed as IgAN in the world. The natural history of IgAN has a relatively poor prognosis; the renal survival rate is 61% at 20 years after diagnosis (the start of dialysis and renal-related death were used as the end-points of the observation)[15)]. The histological criteria for a diagnosis of IgAN are predominant mesangial IgA deposits with an ordinary association of complement 3 (C_3) deposits. An elevation in serum IgA level is observed in 35~50% of IgAN patients, but the serum IgA level is not always correlated with the severity and activity of the disease[16)]. The high incidence of elevated serum IgA levels may be related to the genetic background of this disease[17)]. Furthermore, the immunological mechanisms implicated in the pathogenesis of IgA deposits have been extensively investigated, yet remain poorly understood[18)19)].

Some studies have investigated the role of T cell-mediated immunity in the development of IgAN. A thymectomy during the neonatal period decreases mesangial IgA deposits but results in the same level of serum macromolecular IgA as that of subjects in the non-thymectomized group[20)]. There is a possibility that endogenous and/or exogenous antigens may be associated with the formation of the immune-complex in IgAN. In this mechanism, T cell-B cell interactions through the ligation of TCR and peptide: MHC are the first step in the immunological cascade.

The aim of the present study was to examine the role of CD28-B7 signaling in the development of IgAN. We selected ddY mice as an animal model for IgAN. These mice have been reported to develop spontaneous mesangioproliferative glomerulonephritis with mesangial IgA deposits and a persistently high serum IgA level[21)]. To achieve this aim, we investigated whether or not the CTLA-4 fusion protein affects the glomerular pathological changes, including IgA deposition and functional parameters, such as serum creatinine and proteinuria.

Materials and Methods

Animals

Six-week-old female ddY mice were purchased from the specific pathogen-free animal facility of Shizuoka Laboratory Animals, Shizuoka, Japan. The animals were housed in autoclaved microisolator cages and fed rodent laboratory chow containing, 21.1% protein, 6.0% fat, 4.2% fiber, 6.4% ash, and vitamins. The chow was made from white fish meal, non-fat milk and soy beans. The animals were given free access to water. All mice were received human care in compliance with the institutional guidelines for the care and use of laboratory animals in research.

Functional analysis

Serum creatinine levels were measured by spectrometry using standard techniques. The mice were placed in metabolic cages for 24 hours with free access to water. After collection, the urine samples were stored at −20 ℃ until protein determination. Urinary protein excretion was measured in duplicate using a Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA, USA) with a bovine serum albumin as the standard.

Light microscopy

As previously described[22], tissue specimens were fixed in 10% neutral phosphate-buffered formalin, embedded in paraffin, and cut into 2-μm thick sections. The sections were stained with periodic acid-Sciff. In the kidney specimen from each mouse, the number of mesangial-endothelial cells per glomerulus were counted and averaged for 30 glomeruli cut through the glomerular hilus.

Immunofluorescent studies

Kidney tissue specimens were snap-frozen at −70 ℃ using n-hexane in a dry ice-acetone bath and then cut into 3-μm thick sections using a cryostat. The sections were stained with FITC-conjugated monospecific goat anti-mouse IgG, IgA, IgM, and C_3 sera (Cappel, Durham, NC, USA) using a direct method. The antisera were tested for monospecificity using immunoelectrophoresis against normal mouse serum. The stained sections were examined, and two or three glomeruli per mouse were photographed under a fluorescence microscope (Olympus AX80, Tokyo, Japan). The intensity of immunofluorescence was graded as negative (point 0), trace (point 0.5), one plus (point 1.0), two plus (point 2.0), and three plus (point 3.0). The assessment was performed by two observers who did not know the background data.

Electron microscopy

Kidney tissue specimens cut for electron microscopy were fixed in phosphate-buffered glutaraldehyde for 2 to 3 hours, postfixed in 2% osmium tetraoxide, dehydrated, and embedded in Epon resin. Ultrathin sections were stained with lead acetate and uranyl acetate and examined under an electron microscope (Hitachi H800, Tokyo, Japan).

Measurement of serum IgA concentration

Blood samples were drawn from the orbital sinus using capillary tubes, and the sera were separated and stored at −80 ℃ until analysis. Serum concentrations of IgA were measured by ELISA using an antibody-pair specifc for mouse immunoglobulin isotype (PharMingen). 2,2-Azino-bis (3-ethylbenz-thiazolne-6-sulfonic acid) was used as a substrate for the horseradish peroxidase-conjugated secondary antibodies, and the absorbance at 405 nm was measured by using a microplate reader (Bio-Rad).

Experimental protocol

Groups of mice (n=4) were given either human CTLA-4 fused with human IgG_1 (CTLA-4Ig) or control human IgG_1. Human CTLA-4Ig and a control human Ig were prepared by utilizing a previously described strategy[23]. The coding sequence for the extracellular portion of human CTLA-4 was joined to the heavy chain constant region C_H1-hinge-C_H2-C_H3 domains of human genomic IgG_1 gene by polymerase chain reaction. The human CTLA-4Ig genetic fusion was cloned into expression vector pNRDSH containing the neomycin-resistance gene for selection in G418 and the DHFR gene for amplification by methotrexate under control of the promoter. Chinese hamster ovary cells were transfected by electroporation. Human CTLA-4Ig was purified by binding to immobilized protein A. The human CTLA-4Ig has been shown to be effective in reducing the severity of experimental autoimmune glomerulonephritis in an animal model of Goodpasture's disease in Wistar-Kyoto rat[24][25]. All treated mice were injected intraperitoneally with a dose of 0.1 mg twice a week for the duration of the study. The mice were sacrificed at 15 or 40 weeks of age.

Statistical analysis

All data are expressed as the mean ± SEM. Differences between groups were determined using the Mann-Whitney U test. An analysis of variance (ANOVA) was used to confirm the differences between multiple data sets.

Results

Before administration, both groups of mice showed no differences in proteinuria (0.38 ± 0.21 mg/day vs 0.39 ± 0.19 mg/day). As shown in Fig. 1A, the mice treated with control human IgG_1 experienced a significant increase in the level of proteinuria (15 weeks of age, 0.45 ± 0.12 mg/day; 40 weeks of age, 1.4 ± 0.35 mg/day; $p<0.01$). In contrast, the mice given CTLA-4Ig showed nearly no change in the level of proteinuria (15 weeks of age, 0.53 ± 0.12 mg/day; 40 weeks of age, 0.67 ± 0.21 mg/day).

No significant difference between the serum creatinine levels of mice treated with control human IgG_1 and those treated with human CTLA-4 Ig was observed at 15 weeks of age (0.32 ± 0.03 mg/dl vs 0.30 ± 0.02 mg/dl) or at 40 weeks of age (0.37 ± 0.05 mg/dl vs 0.35 ± 0.04 mg/dl) (Fig. 1B).

Figure 2 shows the serum IgA levels, which were measured at the time of sacrifice. At 15 weeks of age, no significant difference between the groups was observed (70.7 ± 9.8 mg/dl vs 66.6 ± 8.7 mg/dl). At 40 weeks of age, however, serum IgA levels in mice treated with human CTLA-4Ig (126.9 ± 18.4 mg/dl) were obviously lower than in those treated with control human IgG_1 (360.5 ± 22.8 mg/dl) ($p<0.01$).

The number of mesangial-endothelial cells per glomerulus was also different at 15 and 40 weeks of age (18.6 ± 1.2 vs 32.1 ± 2.9 per glomerulus) in the mice that received control human IgG_1. These changes correspond to the pictures of mesangial proliferative glomerulonephritis at 15 weeks of age (Fig. 3A) and 40 weeks of age (Fig. 3C) that show a marked widening of the mesangial area secondary to an increase in the mesangial matrix. In contrast, mesangial proliferation and expansion were not observed at 15 weeks of age (Fig. 3B) or at 40 weeks of age (Fig. 3D) in mice that were treated with human CTLA-4Ig.

Consistent with the light microscopy findings,

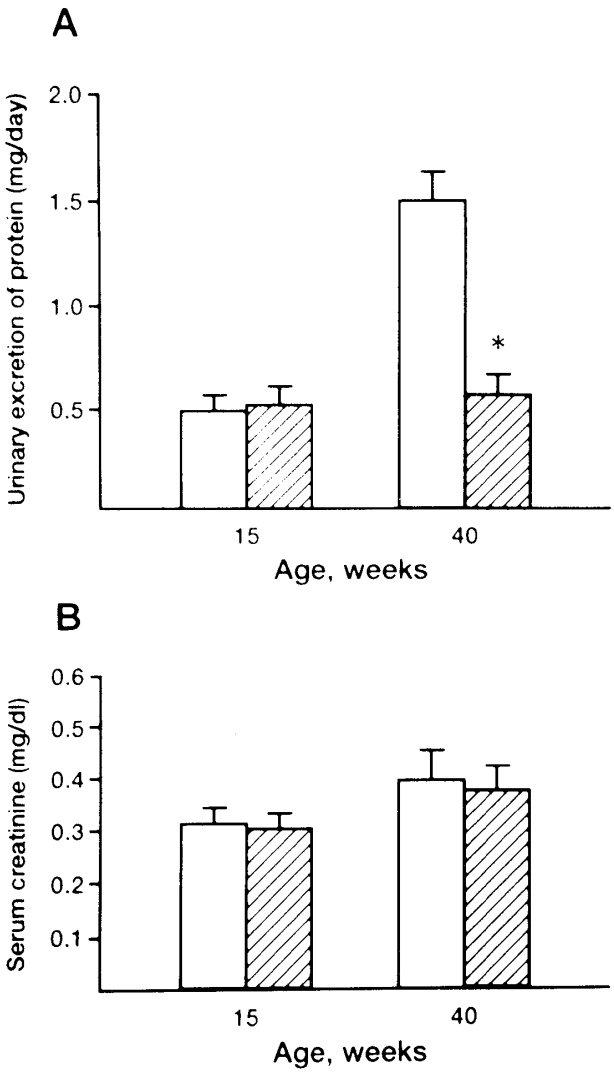

Fig. 1 Urinary protein excretion (A) and serum creatinine (B) in mice treated with control human IgG_1 (open bars) and in mice treated with CTLA-4Ig (closed bars)
Serum and urine were collected from individual mice (n=4) at the indicated time. At 40 weeks of age, mice treated with human CTLA-4Ig showed significantly lower levels of proteinuria than mice treated with control human IgG_1, but there was no significant difference in serum creatinine levels between the two groups. The data are expressed as the mean ± SEM, *: $p<0.01$ vs control.

slight glomerular deposits of IgA were observed in mice of both groups at 15 weeks of age (Fig. 4 A and 4B). Compared with the 40-week-old mice treated with control human IgG_1 (Fig. 4C), 40-week-old mice that were treated with human

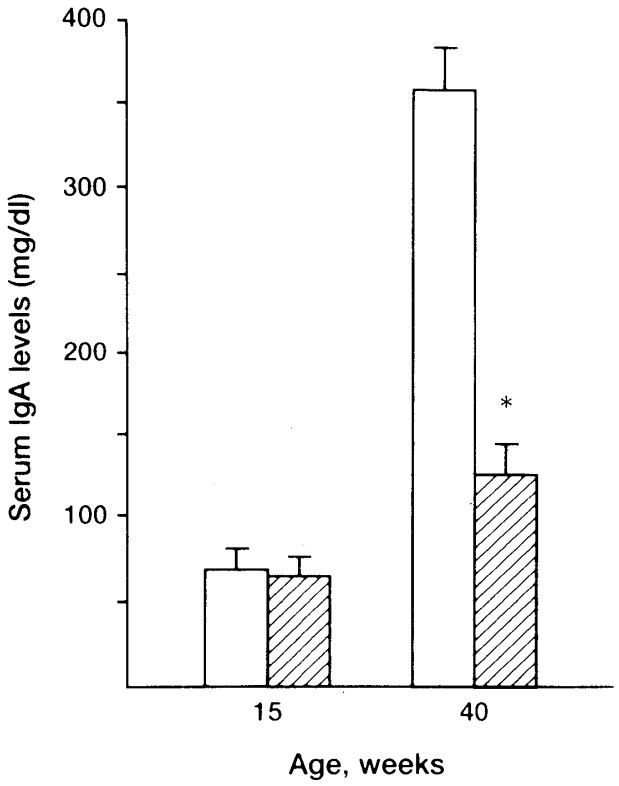

Fig. 2 Serum IgA concentration in mice treated with control human IgG_1 (open bars) and in mice treated with CTLA-4Ig (closed bars)
Serum IgA levels in 40-week-old mice (n=4) treated with human CTLA-4Ig were significantly lower than those of mice given control IgG_1. The data are expressed as the mean ± SEM, * : $p<0.01$ vs control.

CTLA-4Ig showed a marked reduction in mesangial IgA deposits (Fig. 4D). Figure 5 shows the changes in glomerular deposits of IgG, IgA, IgM, and C_3 in both groups. At 15 weeks of age, no difference is observed in the glomerular deposition of IgG, IgA, and C_3 in the two groups. Glomerular deposits of IgG and IgA in mice treated with human CTLA-4Ig diminished in comparison with those in mice given control human IgG_1, while the deposition of IgM and C_3 was the same in both groups.

In 40-week-old mice treated with control human IgG_1, the presence of mesangial cell proliferation as well as electron-dense deposits in the mesangial matrix were clearly observed (Fig. 6A). In contrast, the extent of mesangial cell proliferation decreased, and electron dense deposits were scarcely observed in the mice treated with human CTLA-4Ig (Fig. 6B).

Discussion

Recently, immunosuppressive strategies have been focusing on an important signals via co-stimulatory molecules that affect the clonal expansion of naive T cells. The ligands for CD28 are the B7 molecules found on the surfaces of cells, which are capable of stimulating T cell differentiation or activation. Antigen binding to TCR in the absence of costimulation leads to a state called anergy[26)27)]. Activated T cells express another receptor for B7 molecules called CTLA-4, which resembles CD28 in sequence. However, CTLA-4 binding to B7 molecules delivers an inhibitory signal to the activated T cell. Because B7 molecules are up-regulated in activated APC and CTLA-4 binds to B7 molecules with a stronger affinity than CD28, the interaction of CD28-B7 can be preferentially disturbed using CTLA-4. Taking advantage of this property, CTLA-4 fusion protein has been utilized as a reagent that blocks the interaction between CD28 and B7 molecules. The protein has also been shown to induce antigen-specific T cell unresponsiveness[27)]. CTLA-4 also has important roles in the control of the humoral immune response[28)] and in the suppression of T cell-dependent antibody responses in vivo[26)27)29)]. The early differentiation of naive T cells into interleukin 4 (IL-4) producing Th2 subsets was dependent on CD28 signaling in an autoimmune diabetic model[30)].

Some evidence has suggested that the CD28-B7 interaction is involved in the development of experimental glomerulonephritis. The suppression of CD28-B7 signaling by a human CTLA-4 chimeric protein reduced the severity of anti-glomerular basement membrane (GBM) autoimmune glomerulonephritis in Wistar-Kyoto rats[24)25)]. Moreover, IgG deposition in the glomeruli was

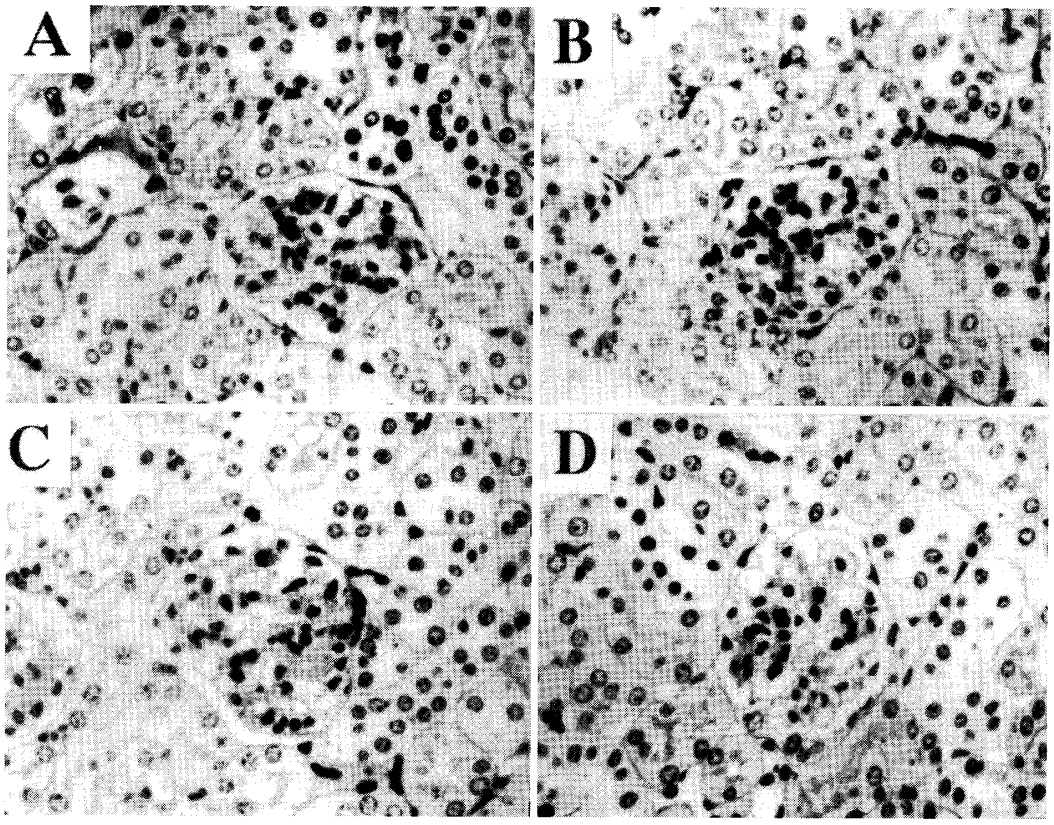

Fig. 3 Representative light micrograph showing mesangial proliferative glomerulonephritis in a 15-week-old mouse treated with control IgG_1 (A). In 40-week-old mice, a marked widening of the mesangial area secondary to an increase in the mesangial matrix is visible (C). In contrast, mesangial proliferation and expansion of the mesangial area is less severe in 15-week-old (B) and 40-week-old (D) mice treated with human CTLA-4Ig. ×400.

markedly decreased, and the degree of glomerulonephritis was significantly reduced in CD28-deficient MRL/lpr mice, which spontaneously develop an autoimmune disorder resembling systemic lupus erythematosus in human[31]. We have recently reported that serum anti-dsDNA titers in the chronic graft-versus-host reaction are not significantly increased in association with no IgG deposition in the GBM of CD28-deficient mice[22]. However, the role of CD28-B7 signaling in the development of human IgAN remains unclear. To address this issue, we have examined the effect of suppressing CD28-B7 signaling using CTLA-4 Ig in ddY mice, a model mouse of IgAN in humans.

In the present study, human CTLA-4Ig was injected intraperitoneally twice a week for the duration of the study. The mean serum IgA level in mice treated with human CTLA-4Ig was approximately 35% of that in mice treated with control human IgG_1. A direct immunofluorescence study showed the reduction of both IgA and IgG deposits in the human CTLA-4Ig group, while IgM and C_3 staining was similar in both groups. The increase and/or the immunological modification of IgA results in an intense mesangial IgA deposition, in which the interaction between T cells and B cells is thought to have a crucial role. Although the process of IgA production in the ddY mice remains unclear, these results clearly indicate that the abnormal mechanism of humoral immunity in ddY mice has something to do with the T cell–B

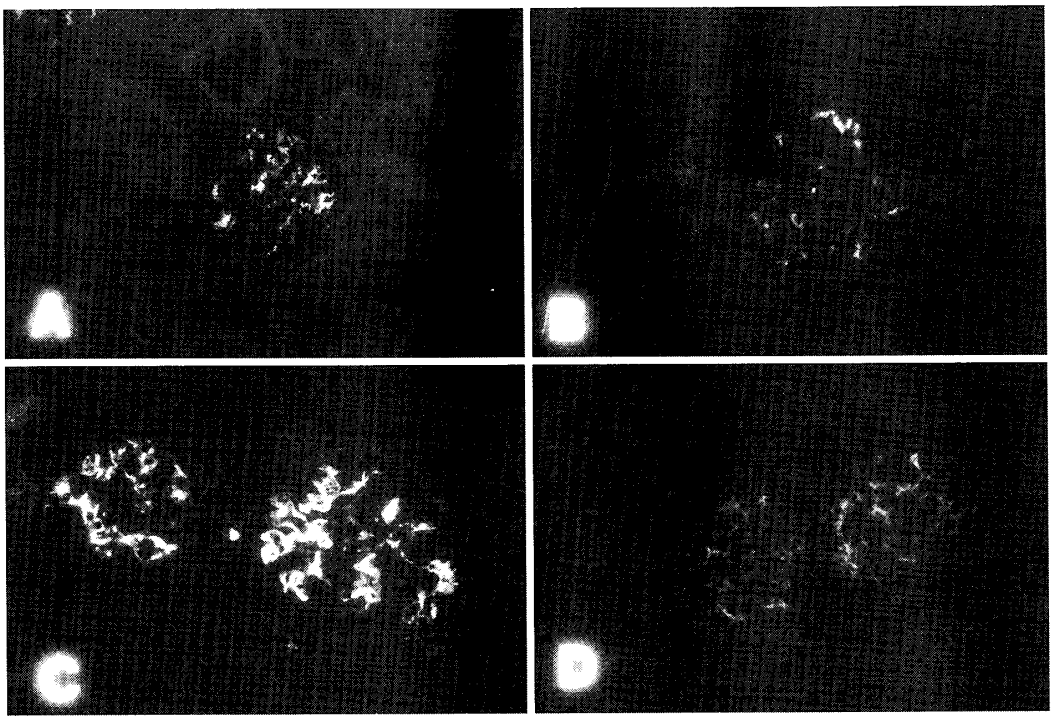

Fig. 4 Results of immunofluorescent staining for glomerular IgA deposition in mice treated with control IgG or CTLA-4Ig
At 15 weeks of age, glomerular IgA intensity is similar in mice given control IgG (A) or CTLA-4Ig (B). Compared to the mouse treated with control IgG (C), the mouse treated with CTLA-4Ig shows a marked reduction in mesangial IgA deposition (D) at 40 weeks of age.

cell interaction. In CD28-deficient mice, immunoglobulin concentrations were only 20% of those in wild-type littermates, and immunoglobulin isotype switching was diminished after injection with some viruses[28]. Our study showed that the inhibition of the production of IgM and the complement system is not effective. This phenomenon corresponds to the result that the serum level of IgG decreased while the level of IgM showed no change in the CD28-deficient MRL/lpr mice[31]. Taken together, blocking interaction between CD28 and B7 could suppress immunoglobulin isotype switching and then reduce mesangial IgA deposition in ddY mice.

In mice treated with human CTLA-4Ig, an increase in mesangial-endothelial cells or the expansion of the mesangial matrix was rarely observed. This result suggests that CTLA-4 protect against lymphocyte and phagocyte migration from the capillary lumen into the glomerular mesangial area. In comparison with healthy ICR mice, the mean number of intraglomerular cells was significantly high in ddY mice treated with anti-CD4 monoclonal antibody (mAb) or control saline solution[32]. Mesangial hypertrophy was milder in ddY mice treated with anti-CD8 mAb than in those treated with anti-CD4 mAb [33]. One possible explanation for this discrepancy is that the degree of glomerulonephritis, including IgA deposition, is not uniform because the ddY mice is a non-inbred strain[21]. In CD28-deficient MRL/lpr mice, glomerulonephritis with mesangial proliferation was less severe than that observed in normal MRL/lpr mice [31]. The infiltration of T cells involves several complicated mechanisms. Reynolds et al[25] reported that CTLA-4Ig amelio-

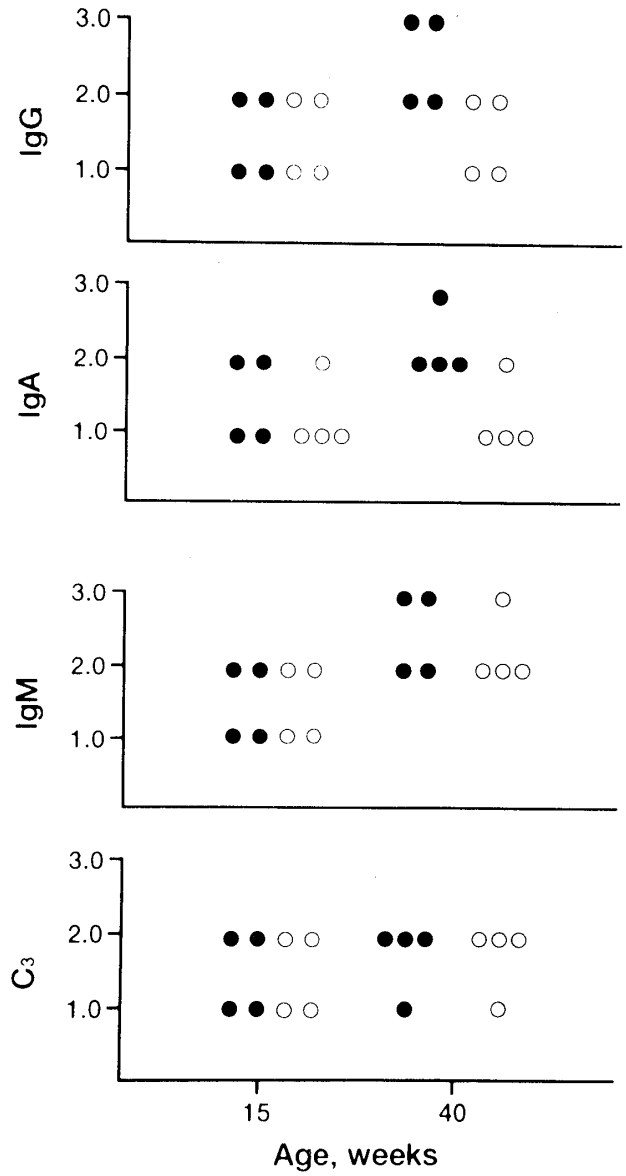

Fig. 5 Scoring for the glomerular deposition of immunoglobulin and C_3 in mice treated with control IgG_1 (●) or CTLA-4Ig (○)
At 15 weeks of age, no difference between the groups is observable. At 40 weeks of age, glomerular IgG and IgA deposits in mice treated with CTLA-4Ig are remarkably lower than those of mice treated with control IgG_1.

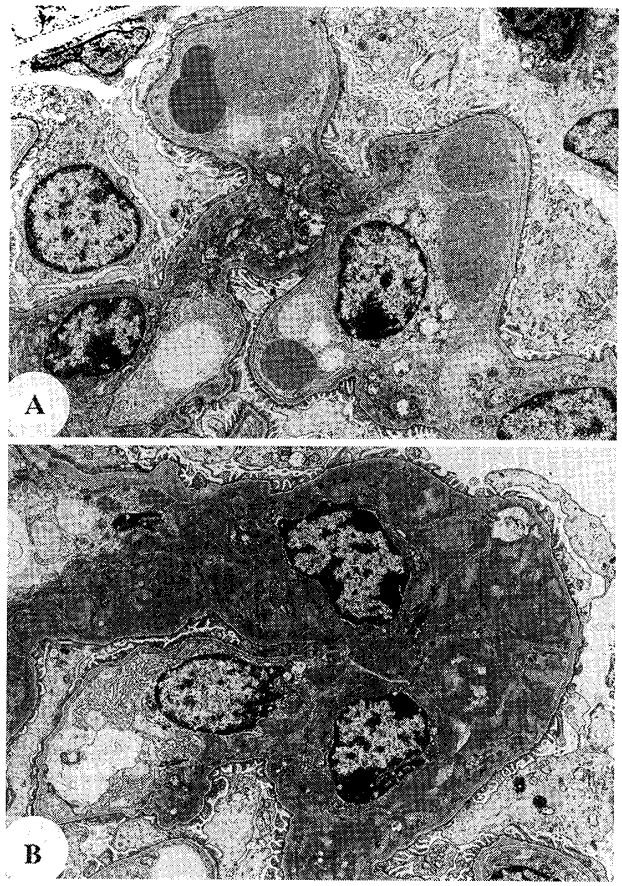

Fig. 6 Electron micrographs of specimens taken from 40-week-old mice treated with human IgG_1 (B) or CTLA-4Ig (A)
In the mouse treated with control human IgG_1, the presence of mesangial cell proliferation as well as electron-dense deposits in the mesangial matrix are clearly observable. In contrast, mesangial cell proliferation appears to a lesser extent and electron-dense deposits are reduced in the mouse that was treated with CTLA-4Ig.

rates rat autoimmune glomerulonephritis by several measures, including the levels of circulating anti-GBM antibodies, the deposition of IgG and fibrin in the glomeruli, the severity of glomerular abnormalities, and the numbers of infiltrating T cells and macrophages. In contrast, a mutant CTLA-4Ig resulted in a similar reduction in the severity of glomerulonephritis, but produced no overall reduction in circulating anti-GBM antibodies, although there was a reduction in IgG2a antibodies. They concluded that CD28-B7 blockade reduces autoantibody production and cellular infiltration of glomeruli. Although the role of costimulatory signals in the homing of T cells is unclear, blocking the CD28-B7 pathway may alter the local factors, such as adhesion molecules and chemokines in ddY mice.

In summary, the results of the present study show that the blockade of CD28-B7 signaling using the CTLA-4 fusion protein induces the decrease in serum IgA levels and proteinuria as well as a reduction in glomerular IgA deposition. We demonstrate for the first time that the development of spontaneously occurring IgA nephropathy can be prevented in ddY mice by blocking the CD28-B7 interaction using a soluble form of CTLA-4Ig. A costimulatory signal via CD28-B7 may play an important role in the development of glomerulonephritis in ddY mice. Although further study is required, CD28-B7 signaling may facilitate the development of therapeutic approaches for human IgAN.

Acknowledgments

We thank Mr. Yasushi Hara for his technical assistance. This study was in part supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (C 11671058).

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ddY マウスに自然発症する IgA 腎症に対する可溶性 CTLA-4 の作用

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本研究は，ddY マウスにおける CD28-B7 を介した co-stimulatory signal の役割を解析することを目的とした．ddY マウスは，ヒトにおける IgA 腎症に類似した糸球体腎炎を自然発症するモデルマウスとして使用されている．今回の研究では，B7.1（CD80）と B7.2（CD86）に結合する CTLA-4（CD152）の fusion protein を使用して，CD28-B7 経路を阻害することにより，本モデルマウスにおける糸球体腎炎が抑制されるか否かについて検討した．各グループ（n=4）に対し，ヒト IgG の Fc ドメインの fusion protein（CTLA-4Ig）またはコントロールとして human IgG_1 を投与した．10～40 週齢までの間，CTLA-4Ig または human Ig を 100μg ずつ週 2 回，腹腔内投与した．15 週齢と 40 週齢の時点で，腎糸球体の IgA 沈着を含む病理学的変化および機能的パラメーターとして血清クレアチニンや蛋白尿を測定した．40 週齢の時点で，human Ig を投与した群は進行性に IgA の顕著な沈着を伴う典型的なメサンギウム増殖性糸球体腎炎を発症した．一方，CTLA-4Ig を投与した群は，メサンギウム増殖性の変化に乏しく，蛋白尿も減少した．免疫蛍光抗体法による観察では，CTLA-4Ig 投与群で糸球体への IgA 沈着の減少が認められた．以上より，今回の研究において，我々は CD28-B7 を介する co-stimulatory signal が IgA 腎症の発症・進展において重要な役割を果たしている可能性を明らかにした．