

Original Article

Anti-Thy-1 Antibody-mediated Complement-dependent Cytotoxicity is Regulated by the Distribution of Antigen, Antibody and Membrane Complement Regulatory Proteins in Rats

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Abstract: Some therapeutic antibodies as anticancer agents exert their effects through the host immune system, but the factors that predict their cytotoxicity, including complement-dependent cytotoxicity (CDC), are unclear. In the present study, we attempted to elucidate some of these factors in a preclinical model. CDC-related mesangiolysis caused by administration of the anti-Thy-1.1 antibody can be studied in the rat anti-Thy-1 glomerulonephritis model, so the model was used in this study. Three animals each were sacrificed at 0.5, 1, 8, 24 and 48 hours after i.v. administration of the anti-Thy-1.1 antibody at 1mg/kg. The distribution of the Thy-1.1 antigen and 2 membrane complement regulatory proteins (mCRPs), Crry and CD55, in three non-treated animals and the distribution of the injected antibody and C3 in the model was studied by immunohistochemistry. In the mesangial cells of the kidney, both expression of the antigen and distribution of the antibody with C3 deposition were observed with weak expression of mCRPs. There was also antigen and antibody distribution in the medullary cells of the adrenal gland and in the lymphocytes of the thymus but no C3 deposition, which was thought to be related to high expression of mCRPs. The antigen was observed in several other organs and tissues without distribution of the antibody. Cell death was only observed in the mesangial cells. These results clearly demonstrate that activation of CDC is regulated by several factors, such as distribution of the target molecule, antibody distribution and the balance among the molecules of the CDC cascade and mCRPs. (DOI: 10.1293/tox.26.41; J Toxicol Pathol 2013; 26: 41–49)

Key words: antibody, complement-dependent cytotoxicity, antigen, membrane complement regulatory protein, rat anti-Thy-1 glomerulonephritis model

Introduction

Antibodies provide a swift solution to therapeutic targeting of disease-related molecules that are discovered through genomic research. The antibodies are raised against many kinds of molecules and exert their efficacy through their various natural functions. While many of these antibodies block the physiological function of their target antigens by neutralization, therapeutic antibodies with complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC) or that act as drug delivery carriers (missile therapy) have also been launched as anticancer agents¹.

The cell death induced by antibodies through CDC depends on the antigen expression levels in the target cells *in vitro*². On the other hand, there are conflicting data con-

cerning the dependence of cell death on antigen expression levels in target cells *in vivo*^{3–5}, but the reasons are not clear. One of the reasons may be the presence of membrane complement regulatory proteins (mCRPs) that are reported to induce resistance against the biological reaction of antibodies^{6,7}. Thus a model suited for studying the factors that predict the efficacy or toxicity of CDC-type therapeutic antibodies is necessary to address these matters.

The anti-Thy-1 glomerulonephritis model is well known as an animal model for the involvement of antibody-mediated CDC in the induction of tissue injury^{8,9}. Thy-1 was originally identified as a lymphocyte differentiation marker in mice and is expressed in several organs, such as mesangial cells in the kidney, lymphocytes, neurons and many other cells^{10–15}. Although the antigen is widely distributed in various tissues, cell death induced by the anti-Thy-1 antibody only occurs in mesangial cells¹⁶. Thus it is suggested that the mCRPs expressed in cells with antibody distribution may be related to the inhibition of cell death in the rat anti-Thy-1 glomerulonephritis (rat anti-Thy-1) model. This evidence suggests that systemic analysis of this model may be useful for addressing the factors that predict the activation of CDC, including complement regulators *in vivo*.

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From this view point, in the present study, we first examined the distribution of the Thy-1.1 antigen and mCRPs in non-treated animals and then the distribution of the injected antibody and C3 deposition in the rat model by immunohistochemistry. As mCRPs, we selected and analyzed complement receptor 1-related gene/protein Y (Crry) and decay-accelerating factor (CD55) because of their broad distribution and their ability to inhibit C3 convertases that are key to the reaction of antibody-induced CDC^{17–21}.

Materials and Methods

Animals

A total of 24 male Wistar rats at 6 weeks of age were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used in this experiment at 7 weeks of age. They were housed in wire cages in an environmentally controlled room (temperature of $23 \pm 3^\circ\text{C}$, relative humidity of $55 \pm 20\%$, ventilation rate of 10–16 times per hour and 12-h/12-h light/dark cycle), fed pelleted chow (CE-2; Clea Japan, Inc., Tokyo, Japan) and tap water *ad libitum*. Animals were sacrificed by exsanguination under anesthesia for pathological examination. All experiments on the animals were approved by the Ethical Committee for Treatment of Laboratory Animals at Chugai Pharmaceutical Co., Ltd.

The rat anti-Thy-1 model

Rats were given intravenous injections of a monoclonal anti-Thy-1.1 antibody (OX-7, mouse, Cedarlane Laboratories Ltd., Burlington, ON, Canada) solution diluted with phosphate-buffered saline (PBS) at 1mg/kg body weight, as described previously^{22,23}. The animals were sacrificed at 0.5, 1, 8, 24 or 48 hours after treatment. The kidney lesions in the present model start from early changes including karyolysis, mesangiolytic changes and ballooning of the capillary loop within 24 hours after injection of anti-Thy-1.1 antibody, followed by hypercellularity in the mesangium, an increase in mesangial matrix during the next few days and finally advance of sclerotic changes²⁴. We aimed to investigate the early changes, so the time points were set at 0.5 to 48 hours. As a control, rats were given an intravenous injection of PBS and were sacrificed at 0.5 or 48 hours after treatment. There were 3 animals per time point for the antibody-injected and control groups. To determine the distribution of the Thy-1.1 antigen, Crry and CD55 in the normal rat, 3 non-treated animals were also used.

Tissue preparation

The kidney was selected according to high expression of Thy-1 in the glomerular mesangial cell¹². Lymphoid organs and organs of the nerve system show high expression of Thy-1^{10,11,13,14,25}. Thus lymphoid organs, including the thymus, spleen and mesenteric lymph node, and organs of the neuroendocrine system, including the adrenal gland, cerebrum and sciatic nerve, were selected. In addition, the lung, liver and heart were also examined. At necropsy, the kidney, adrenal gland, thymus, cerebrum, sciatic nerve,

spleen, lung, mesenteric lymph node, liver and heart were removed from each animal. The tissues were processed and embedded in paraffin by the PLP-AMeX method²⁶ or were frozen in OCT compound. Tissue sections were cut at 3–5 μm from the paraffin blocks or frozen tissue and used to prepare HE-stained sections and for immunohistochemical staining for histological evaluation.

Immunohistochemical staining for Thy-1.1, Crry, CD55 and C3

In non-treated animals, immunohistochemistry for Thy-1.1 was carried out in all collected organs and for Crry and CD55 in the kidney, adrenal gland and thymus. In the rat anti-Thy-1 model, the kidney, adrenal gland and thymus were examined for C3. Antibodies against Thy-1.1 (CD90, OX-7, Cedarlane Laboratories Ltd., Burlington, ON, Canada, 10 $\mu\text{g}/\text{mL}$), Crry (512, BD PharMingen, San Jose, CA, USA, 0.7 $\mu\text{g}/\text{mL}$), CD55 (I-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, 8 $\mu\text{g}/\text{mL}$) and C3 (Cappel, Aurora, OH, USA, 10 $\mu\text{g}/\text{mL}$) were used as the primary antibodies and applied to tissues processed by the PLP-AMeX method. Isotype- and species-matched antibodies were used as negative controls. Immunohistochemical staining was performed according to the labeled streptavidin-biotin (LSAB) method with a Dako LSAB kit (Dako Denmark A/S, Glostrup, Denmark). Antigen retrieval for Thy-1.1, Crry and CD55 by microwave heating in 0.01 M citrate buffer (pH 6.0) at 98°C in a microwave oven (H2800; Energy Beam Sciences, East Granby, CT, USA) was performed prior to applying the primary antibody. The immunoreaction was visualized by a peroxidase-diaminobenzidine reaction. The sections were counterstained with hematoxylin.

Immunohistochemical staining for the injected anti-Thy-1.1 antibody

To analyze the distribution of the injected anti-Thy-1.1 antibody, an antibody to mouse immunoglobulin (Dako LSAB kit, as above) was applied as the primary antibody for immunohistochemical detection of the injected antibody in all collected organs. Frozen tissues were immunohistochemically stained according to the LSAB method and then visualized and counterstained as described in the previous paragraph.

Histopathological evaluation

The changes related to cell death were examined in HE-stained sections prepared from PLP-AMeX-processed tissues. To analyze the distribution of Thy-1.1, Crry, CD55 and the injected anti-Thy-1.1 antibody, the intensity and frequency of immunohistochemical reactivity were evaluated. The intensity was graded as negative, weak, moderate or strong. The frequency of positive reaction in the assessed area was graded as 0%, 25%, 50%, 75% or 100%. For C3 distribution, the immunohistochemical reactivity was judged as negative or positive.

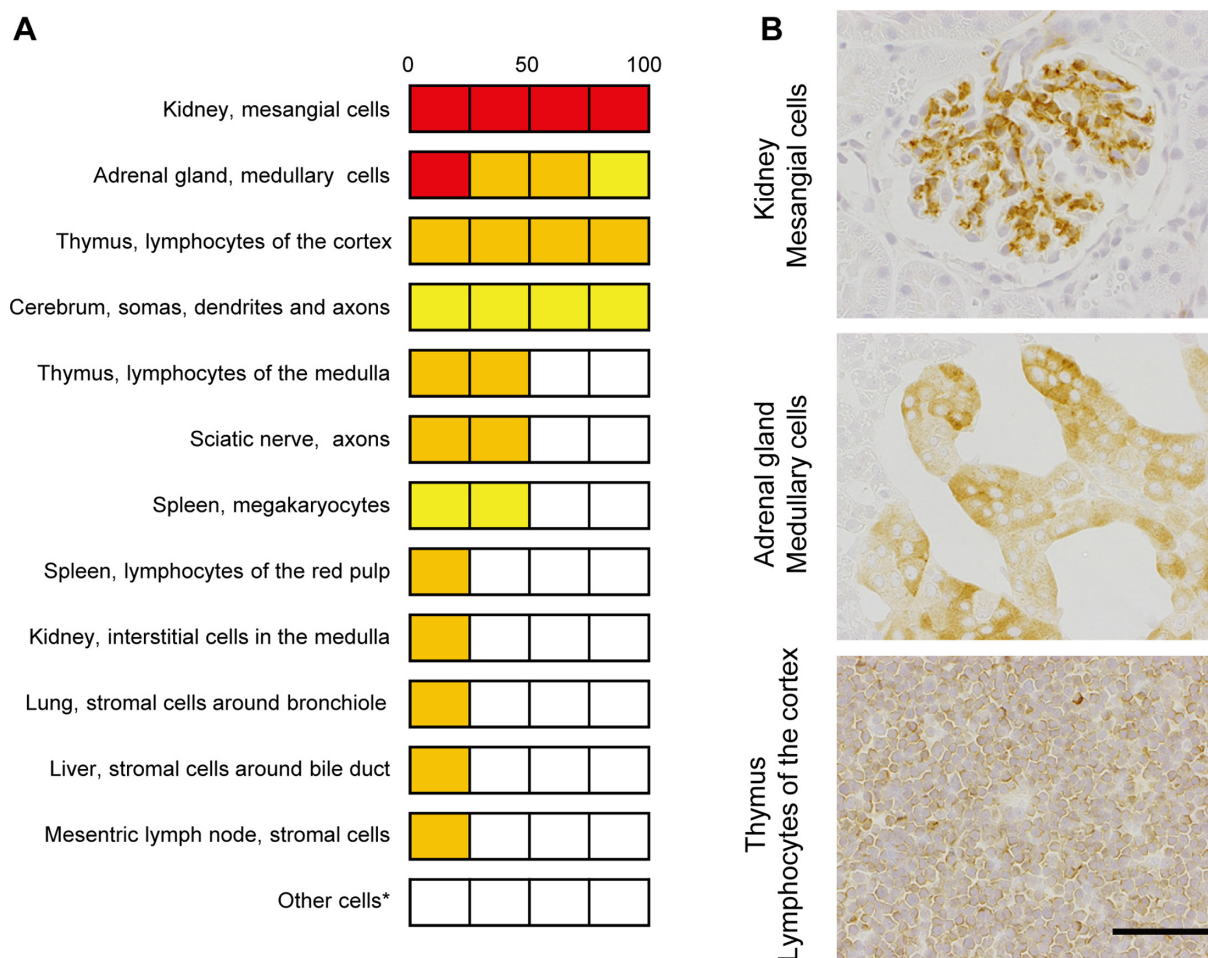


Fig. 1. Distribution of Thy-1.1 antigen in normal rats. A) The distribution of the Thy-1.1 antigen is shown by the intensity and frequency of positive staining. Red, orange, yellow and white indicate strong, moderate, weak and negative staining intensity, respectively. The frequency of staining is shown as the number of colored columns. Each column stands for 25%. *Other organs and tissue elements. B) Immunohistochemistry of Thy-1.1 in the kidney, adrenal gland and thymus. Positive reactions can be seen in mesangial cells of the kidney, medullary cells of the adrenal gland and lymphocytes of the cortex in the thymus. Bar = 50 μ m.

Results

Distribution of the Thy-1.1 antigen in normal rat

The Thy-1.1 antigen was widely distributed in rat organs and tissues (Fig. 1A). Strong staining was only observed in mesangial cells in the kidney and medullary cells of the adrenal gland (Fig. 1A and B). In the medulla of the adrenal gland, the intensity was heterogeneous and ranged from weak to strong (Fig. 1A and B). Moderate staining in the thymus was mainly noted in lymphocytes of the cortex (Fig. 1A and B). In the nerve system, positive reactions were noted in somas, dendrites and axons in the cerebrum and axons of the sciatic nerve (Fig. 1A). In the spleen, megakaryocytes and lymphocytes of the red pulp showed weak to moderate staining (Fig. 1A). Positive reactions were seen in some of the interstitial cells in the medulla of the kidney and in stromal cells of the lung, liver and mesenteric lymph node (Fig. 1A). No positive reaction was noted in any of the other organs or tissues (Fig. 1A).

Distribution of externally injected anti-Thy-1.1 antibody

While the anti-Thy-1.1 antibody was not detected in the kidneys of control rats given PBS (Fig. 2), antigen-specific and nonspecific distributions were observed in the rat anti-Thy-1 model injected with the anti-Thy-1.1 antibody. Antigen-specific distribution was found in mesangial cells of the kidney, medullary cells in the adrenal gland and lymphocytes in the thymus (Fig. 2). The antibody was distributed in all the mesangial cells; however, in the adrenal gland and thymus, antibody distribution was limited to the medullary cells located near the corticomedullary junction of the adrenal gland and in the perivascular area of the cortex in the thymus (Fig. 2). The antibody was detected at 0.5 hours after injection, with no increase observed until 48 hours after injection. Specific distribution was otherwise scarce and only found in lymphocytes of the medulla in the thymus and red pulp of the spleen. The antibody was not found in the other Thy-1.1-positive cells, such as the other lymphocytes in the

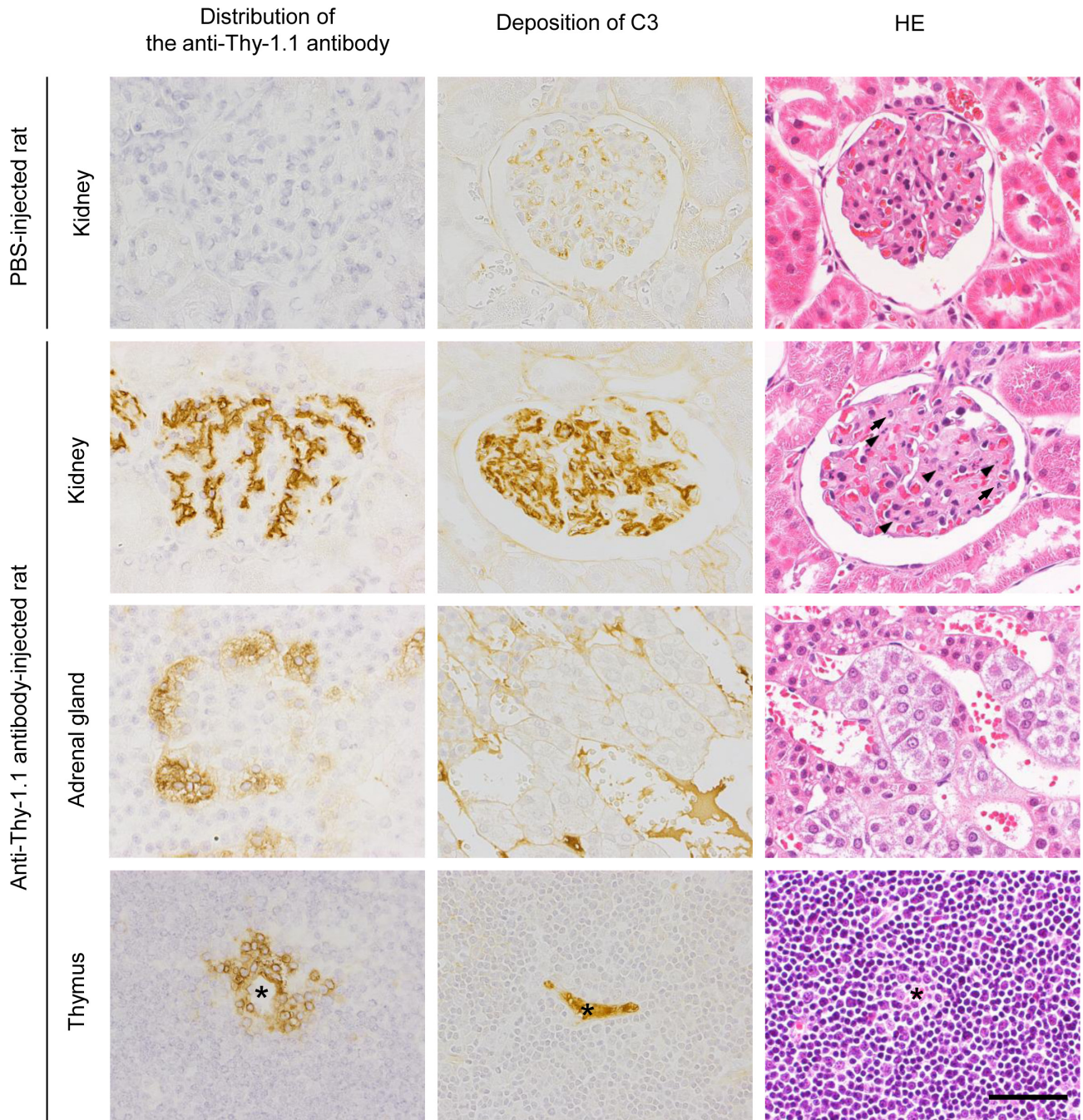


Fig. 2. Immunohistochemical and histopathological findings in the PBS- or anti-Thy-1.1 antibody-injected rats. Immunohistochemistry for the anti-Thy-1.1 antibody and C3 and HE staining are shown. Distribution of the injected anti-Thy-1.1 antibody can be seen in the kidney, adrenal gland and thymus of the antibody-treated rat but not in the PBS-treated animal. C3 deposition in the mesangial cells of the kidney can be seen in the antibody-treated animal but cannot be seen in the PBS-treated animal. There is no positive staining in the adrenal gland or thymus of the rat injected with anti-Thy-1.1 antibody. Mesangial cell death can be seen even though cell death cannot be seen in the adrenal gland and thymus of the rat injected with anti-Thy-1.1 antibody. Karyolysis in the mesangial cell (arrowheads) and infiltrations of a small number of neutrophils (arrows) in anti-Thy-1.1 antibody-injected rat are also shown. The asterisks indicate the blood vessel in the thymus. All images are from animals at 0.5 hours after injection of PBS or the antibody. Bar = 50 μ m.

thymus and the other medullary cells of the adrenal gland. Nonspecific distribution of the anti-Thy-1.1 antibody was observed in Kupffer cells of the liver, macrophages and endothelial cells, which are all cells that express Fc receptors.

C3 deposition and cell death induced by the anti-Thy-1.1 antibody

C3 was detected in the serous substance seen in blood vessels in both PBS- and anti-Thy-1.1 antibody-injected rats (Fig. 2). In control rats given PBS, C3-positive staining

Hours after injection of the anti-Thy-1.1 antibody

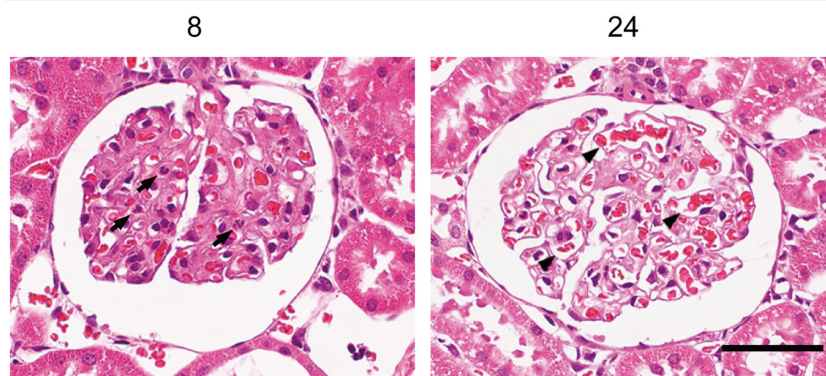


Fig. 3. Changes in the mesangial cells of the kidney at 8 and 24 hours after injection of the anti-Thy-1.1 antibody in HE staining. In the animal sacrificed at 8 hours after injection, the number of mesangial cells is decreased with reduced karyolysis and increased neutrophil infiltration (arrows). In the animal sacrificed at 24 hours after injection, the mesangial area is decreased and is accompanied by capillary dilatation of the glomerulus (arrowheads). Bar = 50 μ m.

was not observed in mesangial cells, and no morphological changes were seen at 0.5 and 48 hours after injection (Fig. 2), but in the rat anti-Thy-1 model, C3-positive staining and cell death were noted in mesangial cells of the kidney (Fig. 2) as early as 0.5 hours after injection of the antibody. Morphologically, karyolysis in the mesangial cells and infiltration of a small number of neutrophils were found at 0.5 and 1 hour after treatment (Fig. 2). At 8 hours after injection, the number of mesangial cells was decreased, with reduced karyolysis and increased neutrophil infiltration (Fig. 3). At 24 and 48 hours after injection, in addition to the reduction in mesangial cell number, the mesangial area was decreased and accompanied by capillary dilatation of the glomerulus (Fig. 3).

On the other hand, other cells with specific distribution of anti-Thy-1.1 antibody, namely medullary cells of the adrenal gland and lymphocytes in the thymus, showed neither C3 deposition nor cell death (Fig. 2). Neither C3 deposition nor cell death was observed in any of the other organs or tissues that were evaluated at 0.5 to 48 hours after injection.

Normal expression levels of mCRPs in the kidney, adrenal gland and thymus

As explained above, antigen-specific distribution anti-Thy-1.1 antibody was only found in mesangial cells of the kidney, medullary cells of the adrenal gland and lymphocytes of the thymus, although Thy-1.1 antigen was widely distributed in rat organs and tissues. Cell death was observed only in mesangial cells in the kidney; it was not observed in medullary cells of the adrenal gland or lymphocytes of the thymus. Thus the kidney, adrenal gland and thymus were selected and evaluated for the expression of mCRPs. As a result, the expressions of mCRPs were found in glomerular cells of the kidney, medullary cells of the adrenal gland and lymphocytes of the thymus. In the kidney, weak expression

of Crry and no expression of CD55 were observed in the mesangial cell, while CD55 was found in podocytes (Fig. 4). In the adrenal gland, weak expression of Crry and strong expression of CD55 were observed in medullary cells (Fig. 4). In the thymus, moderate, diffuse expression of Crry and no expression of CD55 were seen in lymphocytes (Fig. 4).

Discussion

Thy-1 (CD90) is a 25-35-kDa, GPI-anchored cell surface protein²⁷⁻²⁹. There are two alleles in mice that code for Thy-1.1/CD90.1 and Thy-1.2/CD90.2, and they differ by one amino acid²⁹. Thy-1 is involved in T cell activation, neurite outgrowth, apoptosis, tumor suppression, wound healing, fibrosis and multiple signaling cascades³⁰. In this study, we demonstrated the broad distribution of the Thy-1.1 antigen, including mesangial cells of the kidney, medullary cells of the adrenal gland, lymphocytes of the thymus and spleen, somas, dendrites and axons of the cerebrum, axons of the sciatic nerve and stromal cells in several organs. These results are consistent with a previous report that shows Thy-1 expression in various cell types^{10-15,25,31,32}. Therefore, we expected that the rat model generated by external injection of the anti-Thy-1.1 antibody would be a good model to investigate the factors other than antigen expression that predict the activation of CDC.

To this end, detailed histopathological evaluation was performed in the rat anti-Thy-1 model. Interestingly, cell death induced by the anti-Thy-1.1 antibody was observed only in mesangial cells. The histopathological findings of mesangial cells were characterized by karyolysis, neutrophil infiltration and decreased mesangial area and were similar to those in a previous report²⁴. The complement system eliminates pathogens and infected cells through three main activation pathways —the classical, lectin (MBL/ Fi-

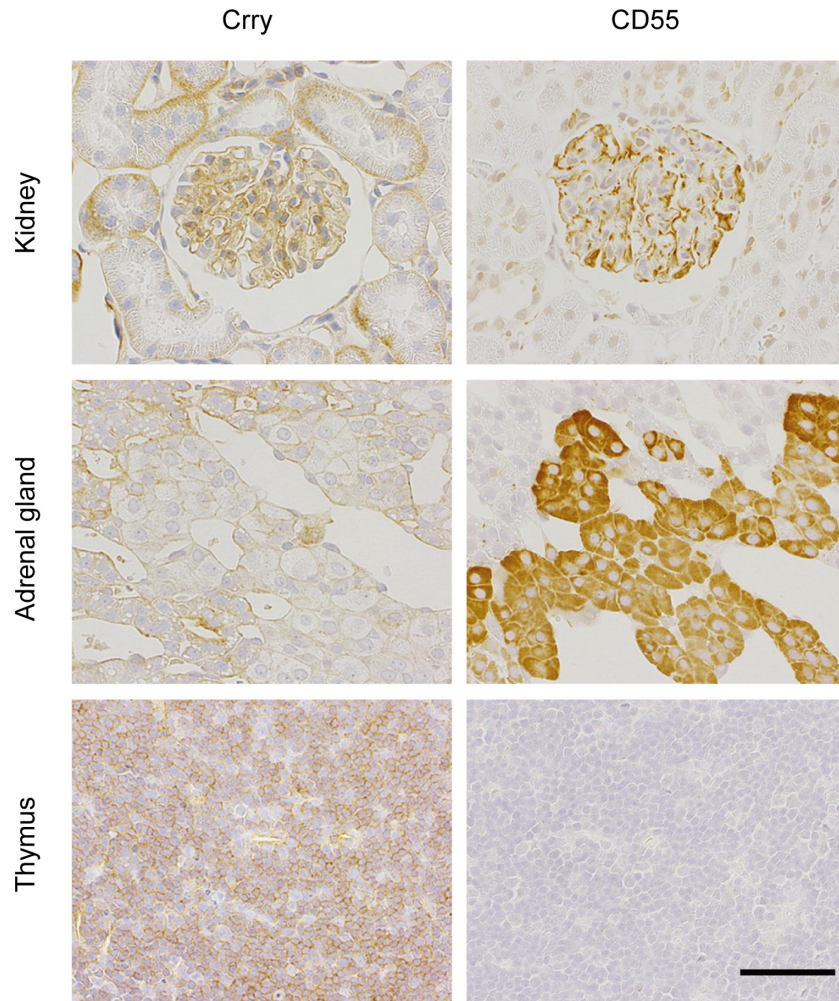


Fig. 4. Expression of mCRPs in mesangial cells and podocytes of the kidney, medullary cells of the adrenal gland and lymphocytes of the thymus in normal rat. Immunohistochemistry of Crry and CD55 is shown. Bar = 50 μ m.

colin) and alternative pathways. Antibody-mediated CDC is activated via the classical pathway and is initiated by the binding of C1q to either IgG or IgM bound to antigen in the immune complex³³. C3 is a key molecule of the complement cascade playing a critical role in CDC activated through the classical pathway *in vivo*. In this study, C3 deposition was seen in mesangial cells at 0.5 hours after injection of the anti-Thy-1.1 antibody. Judging from the results of histopathological examination and C3 deposition, cell death of mesangial cells was induced by CDC mechanisms, as previously reported^{8,9,24}. Although antibody-induced CDC was observed in the mesangial cell, some of the organs and tissues that express Thy-1.1 did not show cell death in this model. This result indicated that the antigen distribution data alone is not sufficient to predict the induction of antibody-mediated CDC.

Keeping in mind that Thy-1.1 antigen distribution was not consistent with cell death, we next considered whether the injected antibody was distributed in organs and tissues expressing Thy-1.1. The anti-Thy-1.1 antibody was distributed rapidly in adequate amounts at 0.5 hours after injection,

and there was no increase at 48 hours after injection. It has been reported that the distribution of injected rat anti-Thy-1 antibody was limited to areas around cortical capillaries in the mouse thymus³⁴. Thus, we evaluated the distribution of the injected anti-Thy-1.1 antibody in organs and tissues with Thy-1.1 expression. The injected anti-Thy-1.1 antibody was distributed in the medullary cells located in the corticomedullary junction of the adrenal gland and the perivascular areas of the cortex in the thymus, in addition to the mesangial cells of the kidney. These results indicate that the injected anti-Thy-1.1 antibody did not bind to all of the cells that expressed the antigen but was limited to some cells that expressed more than a certain level of antigen.

In a preliminary study in rats treated with 5 mg/kg of the anti-Thy-1.1 antibody, the distribution in cells was limited in a similar way to that of the 1mg/kg treatment, although the distribution was broader in the adrenal gland and thymus. The reason for the limited localization in the organs and tissues with Thy-1.1 expression was unclear; however, it has been reported that the structure of each organ, including the vascular structure, can affect the distribution of injected

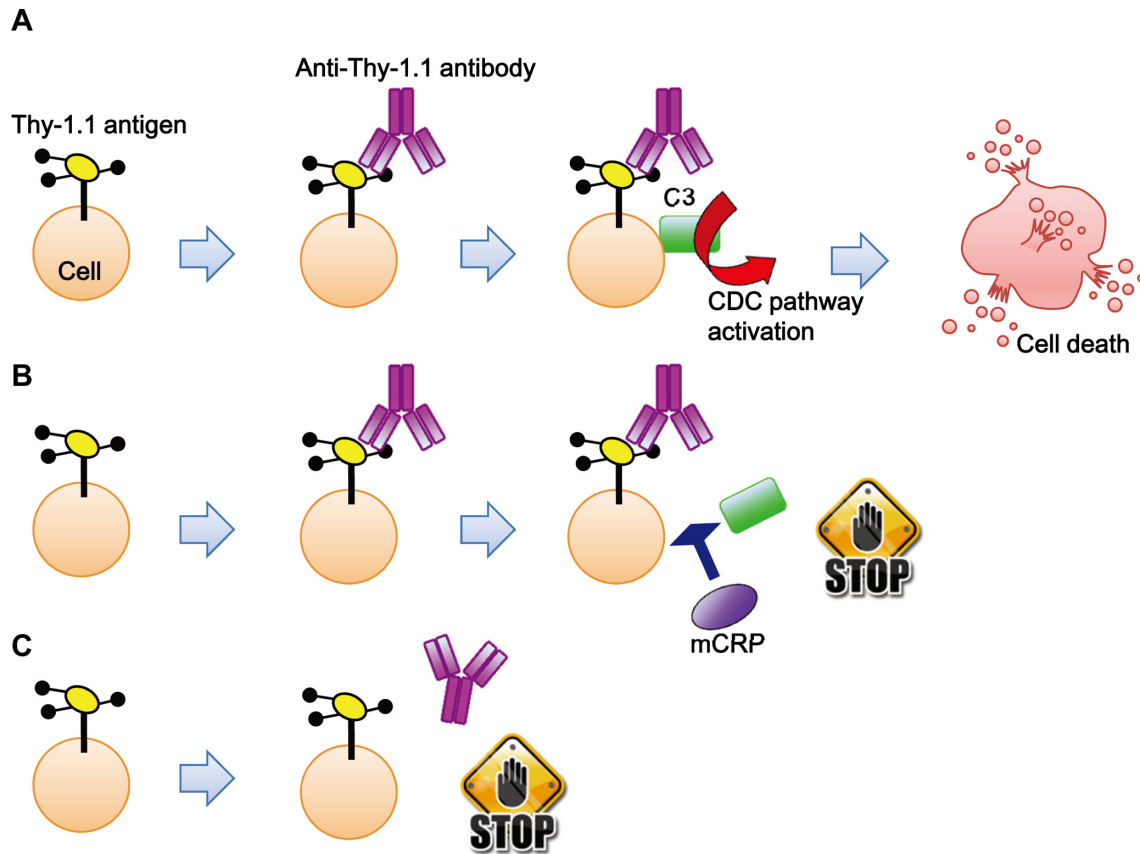


Fig. 5. Categories of CDC activation in Thy-1.1 antigen-expressing cells. A) The antibody binds to the antigen, which leads to activation of the CDC pathway and then to cell death. B) The antibody binds to the antigen, but the CDC pathway is not activated because of mCRP expression. Cell death is not induced. C) The antibody does not bind to antigen, and the CDC pathway is not activated; thus, and there is no cell death.

antibodies³⁵. The distribution in the present model may also be affected by similar mechanisms.

Through our results concerning antigen expression, antibody distribution and cell death, the relationship between antigen-antibody binding and CDC activation were categorized into the following three types (Fig.5): A) antigen-antibody binding that causes cell death (mesangial cells of the kidney); B) antigen-antibody binding that does not induce cell death (medullary cells in the adrenal gland and lymphocytes of the thymus); C) no antigen-antibody binding and no cell death. There were definite differences in C3 deposition between type A and type B cells. In other words, C3 deposition was observed in mesangial cells, which showed cell death, but was not seen in medullary cells in the adrenal gland and lymphocytes of the thymus, which did not show cell death. These results suggest that regulatory factors are related to CDC induction.

Many complement regulatory systems have been reported in the complement activation pathways. It is generally considered that soluble/secreted complement regulatory proteins and mCRPs are important factors for CDC regulation in the classical pathway, especially at the point of C3 deposition, because this molecule is a central component of the complement system^{21,36,37}.

In this study, Crry and CD55 were selected and analyzed because of their broad distribution and ability to locally inhibit C3 convertases¹⁷⁻²¹. In the present study, we examined their distribution and relationship with cell death. As a result, mesangial cells, the only cells to show cell death, were found to express Crry weakly and were negative for CD55. Medullary cells in the adrenal gland showed weak expression of Crry and moderate expression of CD55. Lymphocytes in the thymus showed moderate expression of Crry and no expression of CD55. Thus, cells with antigen-antibody binding that does not induce cell death (Fig. 5B) express relatively high levels of Crry or CD55.

In the mesangial cells, expression of Crry was observed but cell death was induced by antigen-antibody binding. Although the reason for this was unclear in the present study, it has been reported that injection of a combination of antibodies to Crry and Thy-1.1 led to more severe mesangial lysis and leukocyte infiltration compared with administration of only the anti-Thy-1.1 antibody³⁸. Thus we considered that low levels of expression are biologically significant but not sufficient to completely inhibit CDC. On the other hand, high expression of Crry and CD55 was seen in cells with antigen-antibody binding and no cell death. Therefore, we considered that mCRPs are involved in the suppression of

CDC and that a sufficient level of mCRP expression is required to inhibit CDC. Thus, since the expression of mCRPs and C3 deposition seem to be some of the key factors (besides antibody distribution) that determine the activation of CDC, analysis of these molecules may be effective for predicting the biological response to therapeutic antibodies *in vivo*.

In the process of therapeutic antibody development, various regulatory agencies require tissue cross-reactivity studies using immunohistochemical technology when applying for entry into human, to identify potential target organs and tissues for toxicity³⁹. In addition to this, antibody engineering techniques have progressed, and it is now possible to create antibodies that induce stronger host immune responses. These advances also increase the need for research to understand the role and regulation of the factors that predict the response in the biological context. In the current study, we demonstrated that the biological response to an antibody through a CDC mechanism is regulated not only by the distribution of the target molecule but also by various aspects, from antibody distribution to the host immune system (Fig.5). Preclinical research that matches the mode of action of each therapeutic antibody in this way may contribute to understanding the risks and benefits of the therapeutic antibody in the clinical setting.

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