Lung edema and thrombosis induced by activated mannose-binding lectin-associated serine protease 2 and MHC class I reactions, and liver thrombosis aggravated by suppressed phosphotyrosine and female donor reactions to male-specific antigens in Lewis rats

OBJECTIVE The causes of lung edema and thrombosis and liver embolism were investigated in rats. METHOD Lewis rats were used. Mannose-binding lectin (MBL)-associated serine protease 2 (MASP-2) antibody (Ab) was injected to cause lung edema. Lung and liver thrombosis and embolism were generated using major histocompatibility complex (MHC) class I monoclonal Ab (mAb) and MASP-2 Ab. Liver embolisms and male-specific antigen reactions of minor histocompatibility antigens (mHA) were triggered by phosphotyrosine (pTyr) mAb. Correlative expressions of MASP-2 and pTyr were confirmed by flow cytometry (FCM). RESULTS MASP-2 Ab caused acute intraalveolar and peribronchovascular edema, which was characterized by hemolytic red cell casts, type II cell activation with lamellar body (Lb) secretion and elevated apocrine secretion of Clara cells. MHC class I mAb and MASP-2 Ab caused severe lung and liver thrombosis and embolism. Immunocytochemically stained electron micrographs of alveolar type I cells showed a quick-activation of the second MASP-2, when the first MASP-2 was destroyed by MASP-2 Ab. In the liver injected with pTyr mAb, hepatocytes underwent selective apoptosis with Golgi apparatus disturbances. Liver thrombosis, which was aggravated by female donor immune rejections of male-specific mHA, was associated with hepatic venous dilatation. Heparin sodium accelerated lung surfactant (Lb) secretion. Exogenous chemotactic inhibitor suppressed cytokine-induced chemokines, which were triggered by pTyr mAb. CONCLUSIONS Lung edema and thrombosis were caused by MASP-2 and MHC class I Abs. pTyr mAb injection accelerated liver thrombosis, based on tyrosine kinase disturbances, in which mHA immune reactions aggravated liver thrombosis.

Mannose or mannan-binding lectin (MBL)-associated serine protease 2 (MASP-2) belongs to a serine protease family that mediates proteolytic cascades of complement (C), coagulation and fibrinolysis systems. MASP-2 encompasses 2 complement control protein modules (CCP) and a serine protease (SP) domain. MASP-2 dimer functions, as well as the much larger C1r–C1s, tetramer in the C1 complex of the classical pathway through different sets of enzyme-substrate interactions. MASP-2 cleaves C2 and C4. Spontaneous hydrolysis of C3 is initiated by MASP-2. MBL binds to carbohydrate enzyme on the surface of pathogens, and triggers the complement cascade through MASP-2 leading to opsonization. These complement cascades through MASP-2 are associated with innate immune reactions. MASP-2 also promotes fibrinogen turnover after prothrombin is cleaved to thrombin. The activated thrombin splits factor XIII and fibrinogens, and activates platelets.

Stimulation of protein tyrosine kinase results in production of phosphotyrosine (pTyr). Receptor tyrosine kinases (RTK) are important in many biological functions, including
MATERIALS AND METHODS

Animals

Lewis (LEW/SsN) rats were purchased from Japan SLC Co Ltd (Hamamatsu, Japan). Secondarily generated rats, which were born from sibling mothers and the same father, were used in this experiment. They were maintained in the animal center of Hamamatsu University School of Medicine. During their maintenance, microorganism infection was monitored using 2 decoy rats, which were maintained in the same room as the experimental rats. The monitored microorganisms were the hemagglutinating virus of Japan, mouse hepatitis virus, Mycoplasma pulmonis, and B. pili-formis. The results for all of these organisms were negative. The experimental rats were bled and maintained in a clean room.

Experiment designs

This experiment consisted of 6 different systems, classified into experiments (Exps) A, B, C, D, E and F. The detailed experimental designs are summarized in Table 1. These experiments were started using the rat hosts at the ages of 8.1–9.0 weeks. At the time of sacrifice, the body weights (BW) of the experimental female and male rats had increased from 164±5 g to 173±3 g and from 252±3 g to 286±14 g, respectively. Exp F males, which were planned to exhibit graft versus host (GvH) reactions, had BW of 236±4 g at the starting age of 8.1 weeks and 286±14 g at the sacrifice age of 10.8 weeks. Exp C, E and F rats were injected subcutaneously with heparin sodium (Novo Heparin) at a dose of 30 units per rat. Exp C rats were injected with heparin 4 times during 3 subsequent days. Exp E and Exp F rats were injected with heparin 6 times during 7 subsequent days. Rabbit polyclonal antibody (Ab) (IgG) of mannose or mannan-binding lectin (MBL) associated serine protease 2 (MASP-2) were purchased from Santa Cruz Biotechnology, Inc (CA, USA, www.scbt.com). MASP-2 Ab was raised against amino acids 260–344 mapping within an internal region of mouse origin MASP-2. Monoclonal antibody (mAb) (IgG1) of MHC class I was raised against rat class I MHC antigens (RT-IA) to recognize only a subfraction of the total class I MHC molecules (Sera Laboratories International, Ltd, West Sussex, England). Purified mouse anti-phosphotyrosine (pTyr) mAb (IgG2a) had reactivity to human and was tested in mouse, rat, chicken and dog (BD Biosciences, Tokyo, Japan). Chemotactic inhibitor had the structure of N-Boc-Phe-D-Leu-Phe-D-Leu-Phe-OH (Bachem California, Torrance, CA, USA).

Histopathological examination

From each rat, at least a quarter of the lung tissue, 1–1.5 cm×0.8–1 cm-sized pieces of liver, a segment of ileum of 1 cm in length taken from 1 cm above the cecum, and a quarter of the spleen were fixed in 10% formalin (Sigma-Aldrich, St Louis, MO, USA) and stained with hematoxylin-eosin (H-E) for light microscopic examination. Samples from 7 untreated control females (Cont F) and 9 control males (Cont M) were also examined in the light microscopic analysis. A BX51 light microscope with a DP72 digital camera was used for light micrographs. For transmission electron microscopic (TEM) analysis, samples of Exp A-2 lung, Exp B-3 lung and Exp F-2 liver were selected. Immunohistochemical stain was applied before fixing in 2% glutaraldehyde for the detection of MASP-2 antigen or pTyr antigen. Lung samples from Cont F3, Exp C-3 and Exp D-2 and bone marrow (BM) from Exp D-3 were selected for immunohistochemical staining. A lung cell suspension of Cont F3 was divided into two parts. Apart from pTyr detection using the divided lung cell suspension from Cont F3, all the 4 kinds of cell suspensions described above were stained for MASP-2 detection. MASP-2 polyclonal Ab (Santa Cruz Biotechnology Inc) and pTyr mAb (BD Biosciences) were labeled with 15 nm gold colloidal particles (EY Laboratories, Inc, San Mateo, CA, USA) as pre-fixing procedures as described previously. A JEM 12000 TEM (JEOL, Tokyo, Japan) was used to take transmission electron micrographs.

Flow cytometer (FCM) analysis

One million mononuclear cells (MNC) were obtained from mesenteric lymph node (MLN), BM and lung tissue of all the experimental rats. MLN cell suspensions were stained with both
fluorescein-isothiocyanate (FITC) anti-rat CD3 mAb (Cedarlane Laboratories Ltd, Hornby, Ontario, Canada), which recognizes a rat T cell surface antigen located in the periarterial lymphatic sheaths of the spleen, and FITC anti-rat CD8a mAb, which mediates GvH disease in rats (Cedarlane Laboratories Ltd). All the cell suspensions of MLN were incubated with FITC-conjugated mAb for 30 minutes at 4° C. BM cell suspensions were stained with MASP-2 polyclonal Ab (Santa Cruz Biotechnology, Inc), and pTyr mAb (BD Biosciences) for 30 minutes at 4° C, both of which were double-stained with monoclonal mouse IgG1/FITC (Ancell Co, Bayport, MN, USA) for 15 minutes at 4° C. Lung cell suspensions were stained with MASP-2 Ab-FITC in the same way as BM cells. The stained MNC were assayed for their labeled FITC using an EPICSR XL-MCL system III FCM (Beckman Coulter, Fullerton, CA, USA). For the interpretation of MASP-2- and pTyr-positive cells, a fixed point between negative and positive peaks was used, as shown in figures 6a and 6b, because many results did not show a separate negative peak. The percentage of positive cells is presented as mean±standard deviation (M±SD), calculated by the Excel function of STDEVPA.

RESULTS

Table 1 shows a summary of the pathological findings found in the Exp A to Exp F rats. As shown in table 1, Exp A to Exp C rats were given MASP-2 Ab, which caused lung edema. The lung edema of an Exp A-1 female is shown in figure 1a where many hemolytic red cell casts can be observed along with eosinophils infiltrating the arterioles. Small red cell fragments are present around the casts. Hemolytic red cell casts, composed of both hemolytic red cells due to complement activation and red cell aggregation due to hypercoagulability, indicate MASP-2 activation induced by MASP-2 Ab. The lung edema shown by the red cell casts was observed in 75% of Exp A, 80% of Exp B and 100% of Exp C. Seven Cont F and 9 Cont M were examined extensively for red cell casts. In 3 (43%) of the 7 Cont F and 2 (22%) of the 9 Cont M, red cell cast

![Figure 1a. Light microscopy showing an area of edematous lung in Exp A-1 female rat injected with MASP-2 Ab. Many red cell casts, which suggest the occurrence of complement-activated hemolysis, are demonstrated, around which small red cell fragments are present. At the left side, an edematous arteriole is shown, with edematous adventitia infiltrated by many eosinophils. A part of the arteriolar middle tunic, which is composed of smooth muscle, is damaged. MASP-2 Ab reactions to lung are indicated. Magnification indicated at lower right (H-E stain).](image-url)

<table>
<thead>
<tr>
<th>Exp no (n†)</th>
<th>Sex</th>
<th>Antibody</th>
<th>MASP-2</th>
<th>MHC*</th>
<th>pTyr</th>
<th>CI*</th>
<th>BM cell**(x10⁶/rat)</th>
<th>Observation (day)</th>
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<tr>
<td>A (4) F</td>
<td></td>
<td></td>
<td>4 μg</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>0.5−1</td>
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<tr>
<td>B (5) M</td>
<td></td>
<td></td>
<td>4 μg</td>
<td>10 μg</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>0.5−1</td>
</tr>
<tr>
<td>C (4) M</td>
<td></td>
<td></td>
<td>5 μg</td>
<td>−</td>
<td>−</td>
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<td></td>
<td>−</td>
<td>−</td>
<td>15 μg</td>
<td>−</td>
<td>−</td>
<td>0.5−1</td>
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<tr>
<td>E (4) F</td>
<td></td>
<td></td>
<td>−</td>
<td>−</td>
<td>20 μg</td>
<td>−</td>
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<td>−</td>
<td>−</td>
<td>20 μg</td>
<td>−</td>
<td>−</td>
<td>2</td>
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</table>

*Chemotactic inhibitor (150 μg/rat), **Female rat BM cells, †Total rat number, *Class I (RT-IA) mAb, §Heparin sodium (30 units/rat) was injected

MASP-2: Mannose-binding lectin (MBL)-associated serine protease 2, MHC: Major histocompatibility complex, pTyr: Phosphotyrosine, BM: Bone marrow

Table 2. Histopathological findings in the rats of 6 Exps A, B, C, D, E and F (illustrated in figures 1−5).

<table>
<thead>
<tr>
<th>Exp no (n*)</th>
<th>Red cell cast n (%)</th>
<th>Vessel edema n (%)</th>
<th>Liver Embolism n (%)</th>
<th>Intestine Activated LN n (%)</th>
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<tr>
<td>A (4)</td>
<td>3 (75)**</td>
<td>4 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B (5)</td>
<td>4 (80)†</td>
<td>5 (100)</td>
<td>3 (60)§</td>
<td>2 (40)</td>
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<tr>
<td>C (4)</td>
<td>4 (100)§</td>
<td>4 (100)</td>
<td>0 (0)</td>
<td>1 (25)</td>
</tr>
<tr>
<td>D (4)</td>
<td>1 (25)</td>
<td>4 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
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<td>0 (0)</td>
<td>4 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
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<tr>
<td>F (5)</td>
<td>1 (20)</td>
<td>5 (100)</td>
<td>1 (20)§§</td>
<td>1 (20)§</td>
</tr>
</tbody>
</table>

*Total rat number, **Figures 1a and 2, †Fig. 3, §Fig. 4, ¶Increased numbers of vacuoles in type II alveolar cells, §§Fig. 5
formations were detected sparsely. In the 2 of the 3 Cont
F and 1 of the 2 Cont M that had red cell casts, eosinophil
infiltration around the blood vessels was also confirmed.
Thus, it appears that mild pre-edematous findings such
as those illustrated in figure 1a also occur physiologically
in relation to eosinophil infiltration even in normal rats.
As shown by immunochemical staining in figure 1b, the
eosinophil granules contained MASP-2-positive complex
antigens. MASP-2 activation must be also triggered by
eosinophil granule degradation. Among the Exp A to Exp C
rats, in which MASP-2 activation was caused by exogenous
MASP-2 Ab injection, rather exceptionally, the Exp A-1 rats
(fig. 1a) had eosinophil infiltration. The eosinophils must
be destroyed by exogenous MASP-2 Ab in these Exp A to
Exp C rats. The electron micrographs shown in figure 2a
and figure 2b obtained from an Exp A-2 rat show signs of
lung edema. Figure 2a demonstrates an activated large
alveolar cell (type II cell). MASP-2 Ab reactions to the type
II cell MASP-2 resulted in lamellar body (Lb) secretion and
formed many large vacuoles in the cytoplasm, which hinted
at the change from type II cell to type I cell. Among Exp A
to Exp C rats, the highest vacuole numbers of type II cells
were observed in Exp C rat tissue sections followed for 3
days after MASP-2 Ab injection. After heparin sodium had
been injected 4 times, heparin sodium induced accelerated

![Figure 1b](image1.png)

**Figure 1b.** Electron micrograph of BM eosinophil derived from Exp
D-3 female rat injected with pTyr mAb. This rat had 78% CD3-positive
mesenteric lymph node (MLN) cells at and 91% MASP-2-positive BM
cells. Eosinophil granules are stained positively with MASP-2 Ab-gold
complexes, many of which have very high density. Eosinophil granules
with MASP-2 antigen complexes are recognized. Magnification indicated
at the lower left (uranyl acetate-lead citrate double-stain).

![Figure 2a](image2.png)

**Figure 2a.** Electron micrograph of lung derived from Exp A-2 female
rat injected with MASP-2 Ab. A large type II alveolar cell in the center
is surrounded by three bundles of reticular fibers. This cell shows many
vacuoles, suggesting that many lamellar bodies (Lb) have already been
secreted from the cell. Forepart from one Lb in the center cytoplasm,
many small-sized Lb are still present at the edge of the cytoplasm and at
center left is an extracellular Lb, which has been secreted from the cell.
This figure shows MASP-2 Ab reactions to the type II cell. Magnification
indicated at lower left (uranyl acetate-lead citrate double-stain).

![Figure 2b](image3.png)

**Figure 2b.** Electron micrograph of lung from Exp A-2 female rat (the same
as shown in fig. 2a), showing a bronchiole consisting almost completely
of Clara cells, which show active merocrine (apocrine) secretion. Between
the apocrine Clara cells, active eccrine secretion is shown. Bronchiole
secretion had been reinforced by MASP2 Ab injection. Magnification
indicated at lower left (uranyl acetate-lead citrate double-stain).
secretion of lung surfactant (Lb) from type II cells to correct lung damage. Figure 2b shows a part of the bronchiole from an Exp A-2 rat, containing Clara cells, which caused active merocrine secretion. The small bronchioles in these rats were often occupied by a large mass of apocrine-secreted Clara cells. Figures 2a and 2b show characteristic lung edema signs triggered by MASP-2 Ab injection. Exp B rats were injected with both MASP-2 Ab and MHC class I mAb, which triggered lung thrombosis and liver embolism. Figure 3 shows two kinds of lung thrombosis generated at the site of the damaged endothelial cells. The thrombosis shown in figure 3a contains apoptotic leukocytes, platelets and fibrins, which had reacted with MHC class I mAb, and underwent apoptosis. In blood circulation, thrombosis was generated at the site of the damaged endothelial cells. As shown in figure 3b, one of the thromboses has the same characteristics as those of figure 3a, but another small thrombosis contains freshly destroyed red cells, caused not only by MHC class I mAb, but also clearly by MASP-2 Ab. Figure 4 shows a liver embolism found in an Exp B-5 rat. The embolism occupied more than a quarter of one hepatocyte. At the embolic site, ischemic hepatocytes were accompanied by segmented neutrophil infiltration. There is a strong possibility that the embolism shown in figure 4 was caused by a lung thrombosis. Another embolism with more severe apoptotic (lysed) hepatocytes accompanied with bleeding was found with a dilated vein near to the embolism shown in figure 4. The latter embolism has the same pathological findings as those of the Exp F-2 male.
described below, which was caused by liver thrombosis. As
the size of the fixed liver samples for tissue section were
small, liver embolism was confirmed in only 3 (60%) of the
5 Exp B rats. However, on examination of the whole liver,
all the Exp B rats appeared to have had liver embolisms
derived from liver thrombosis. At the end of the ileum, the
submucosal LN were remarkably enlarged in 40% of Exp B
rats and 25% of Exp C rats, based on MASP-2 activation.

The Exp D-Exp E and Exp F rats were injected with pTyr
mAbs, as shown in table 1. The Exp E rats were given both
chemotactic inhibitor and heparin sodium for comparison
with the reactions of Exp F males, which were additionally
injected with female BM cells. On light microscopy, the liver
tissue section of Exp F-2 shows a mass of severely apoptotic
(lysed) hepatocytes with bleeding near to a dilated
vein, which was judged to be due to a liver embolism caused
by liver thrombosis. Exp F rat livers showed sporadic single
hepatocyte apoptosis, small focal lymphocyte infiltration
and localized venous-bore dilatation. Around the veins,
hepatocyte damage was generally more severe in the Exp
F rats. Compared with the livers of the Exp E rats, those
of the Exp F rats had signs of being affected not only by
pTyr mAb but also by immune reactions to injected female
BM cells. The electron micrographs shown in figure 5 were
obtained from an Exp F-2 rat. Proliferation and degenera-
tion of Golgi apparatuses are observed in the hepatocyte
of figure 5a. Small apoptotic hepatocytes retain several
degenerated Golgi apparatuses discharged into sinusoidal
lumens, as shown in figure 5b. Whole apoptotic hepatocytes
were often discharged into sinusoidal cavities and venules,
resulting in blockage of the blood flow. In other electron
micrographs of the same liver, moderately or markedly
edematous adventitia of venules was frequently observed,
resulting in narrowing of the vessels.

A small red cell thrombosis containing 4 red cells and 8
red cell fragments in a fibrin net, was detected in the Exp
F-2 male liver. In the lung of Cont F3 stained immunochemi-
cally, the lumen of the Golgi apparatuses contained with
pTyr mAb pTyr-Ab-gold complexes, which caused Golgi
apparatus damage. Heparin sodium at 30 units per rat
had been injected into the Exp E and Exp F rats in both of
which its effectiveness could be expected. For erythrocyte,
platelet or fibrin thrombosis generated in liver venules,
heparin sodium must react to protect the Exp F rats from
liver thrombosis and embolism. Ileum LN enlargement in
the Exp F-5 rat (20%) was as large as that in 2 (22%) Cont
M and 1 (14%) Cont F, but not so much as that found in
the MASP-2 Exp B and Exp C rats.

Table 3 shows the FCM results of MLN, BM and lung

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**Figure 5a.** Electron micrograph of an apoptotic hepatocyte from liver
of Exp F-2 male rat, with acute GvH reactions due to male-specific mHA
after treatments with pTyr mAb, chemotactic inhibitor and female BM
cells. The hepatocytes in the apoptotic area are characterized by prolif-
eration of disturbed Golgi apparatuses. Magnification indicated at lower
left (uranyl acetate-lead citrate double-stain).

**Figure 5b.** Electron micrograph of liver of Exp F-2 male rat (the same
as shown in fig. 5a). Three apoptotic hepatocytes remaining degener-
ated Golgi apparatuses are shown at the upper right. These apoptotic
hepatocytes have been discharged into the sinusoidal lumen (the blood
flow of the sinusoidal lumen is often blocked by discharged apoptotic
hepatocytes). Magnification indicated at lower left (uranyl acetate-lead
citrate double-stain).
Ab. Exp B-3 and Exp C-3 rats, which were injected with MASP-2 Ab or pTyr mAb, showed 92% MASP-2-positive cells in both rats. However, in the 3 rats Exp D-1, Exp E-3 and Exp F-5, which were injected with pTyr mAb, the MASP-2-positive cells were 69%, 89% and 73%, respectively. Apart from the Exp E-3 rat which had exogenous chemotactic inhibitor, Exp D-1 and Exp F-5 rats exhibited more suppressed MASP-2 expression in their lungs. Table 3 summarizes the FCM results of MLN, BM and lung preparations from the six groups of experimental rats (A, B, C, D, E, F) and control rats (Cont) treated with either MASP-2 Ab or pTyr mAb (illustrated in figures 6–7).

Table 3. MNC results of MLN, BM and lung samples from the six groups of experimental rats (A, B, C, D, E, F) and control rats (Cont) treated with either MASP-2 Ab or pTyr mAb (illustrated in figures 6–7).

<table>
<thead>
<tr>
<th>Exp no (Sex)</th>
<th>Total rats (n*)</th>
<th>MLN (%)</th>
<th>BM (%)</th>
<th>Lung (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CD3+</td>
<td>CD8a+</td>
<td>MASP-2+</td>
</tr>
<tr>
<td>A (F)</td>
<td>4</td>
<td>71±2</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>B (M)</td>
<td>5</td>
<td>71±1</td>
<td>NT</td>
<td>99±1</td>
</tr>
<tr>
<td>C (M)</td>
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<td>NT</td>
<td>95±3</td>
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<td>87±8</td>
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<tr>
<td>F (M)†</td>
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<td>68±2</td>
<td>15±1</td>
<td>78±6</td>
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<tr>
<td></td>
<td>1‡</td>
<td>79</td>
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<td>78</td>
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<td>15±1†</td>
<td>99±1†</td>
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<tr>
<td>Cont (M)</td>
<td>7</td>
<td>71±2</td>
<td>15±1</td>
<td>95±6</td>
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*Total rat number, **Not tested, †GvHD, ‡Exp F-3 rat, ††N=3, †††N=5

preparations, which were stained with FITC-conjugated CD3, CD8a, MASP-2 and pTyr mAbs or Ab. In the MLN of Exp D rats that had been injected with pTyr mAb, the CD3-positive cell % was slightly increased to 82±6%. B cell numbers decreased slightly in the Exp D rats. Slightly increased CD8a-positive cells were present in the Exp E rats (18±0.2%) showing 74±3% of CD3-positive MLN cells. Cont F showed 76±3% of CD3-positive MLN cells. Chemokine inhibitor of Exp E rats suppressed CD3-positive MLN cells to 74±3%, from 82±6% found in the Exp D rats without chemotactic inhibitor. Exp F-3 male showed increased CD8a-positive cells in the MLN (23%). The Exp F-3 male had about 50% apoptotic non-lymphocytes in the MLN, and depleted host lymphocyte numbers in the MLN and spleen. Four other Exp F males showed a slightly low % of CD3-positive T cells (68±2%), but no decrease of CD8a-positive cells (15±1%). Thus, in addition to T cell depletion in the MLN, there was a relative increase of CD8a-positive cells in the T cells. Exp F-2, 3 and 5 rats had BW gains of 40 g, 38 g and 42 g, 16 days respectively after the start of the experiment, while the gain for Exp F-1 and 4 rats was 58 g and 71 g, respectively, so the first 3 rats had suppressed BW gain. From the results of FCM of MLN, the Exp F rats were judged to have undergone variable degrees of GvH reaction. Chemotactic inhibitor at 150 μg/rat acted to reduce the signs of GvH reaction. Host lymphocyte depletion from the LN was protected from GvH reaction by chemotactic inhibitor in the Exp F rats, except for the Exp F-3 rat, in which chemotactic inhibitor must react more poorly.

The FCM results of lung MNC stained with MASP-2 Ab conjugated with FITC are demonstrated in figure 6a. MASP-2 antigen was expressed in both type I and type II alveolar cells. The highest % of MASP-2-positive cells was detected in Exp A-3 (97%), which had been injected with MASP-2 Ab. Exp B-3 and Exp C-3 rats, which were also injected with MASP-2 Ab, showed 92% MASP-2-positive cells in both rats. However, in the 3 rats Exp D-1, Exp E-3 and Exp F-5, which were injected with pTyr mAb, the MASP-2-positive cells were 69%, 89% and 73%, respectively. Apart from the Exp E-3 rat which had exogenous chemotactic inhibitor, Exp D-1 and Exp F-5 rats exhibited more suppressed MASP-2 expression in their lungs. Table 3 summarizes the FCM results of MLN, BM and lung preparations from the rats treated with either MASP-2 Ab or pTyr mAb. Exp D, Exp E and Exp F rats exhibited suppressed expression of MASP-2-positive cells (68±2% to 83±5%), while Exp A, Exp B and Exp C rats, which had received MASP-2 Ab, had MASP-2-positive cells at 92±2% to 95±8%. Lung MASP-2 expression was suppressed by pTyr mAb. Exp D rats, without chemotactic inhibitor, exhibited suppressed expression.
(68±2%) of MASP-2-positive lung cells, but Exp E and Exp F rats, with chemotactic inhibitor, showed more mild suppression of MASP-2-positive lung cells. Compared with that of Exp E rats (83±5%), Exp F rats had lower expression of lung MASP-2 (79±4%). It was concluded that chemotactic inhibitor protected Exp E and Exp F rat lungs from MASP-2 suppression, with the help of heparin injections. Figure 6b shows the BM FCM results, stained with FITC-conjugated MASP-2 Ab or with FITC-conjugated pTyr mAb, in the same rats, Exp C-3, Exp E-3 and Exp F-5. In the Exp C-3 rat given MASP-2 Ab, as for the lung FCM, the BM MASP-2-positive cells were 98% and BM pTyr-positive cells were 93%. Exp E-3 and Exp F-5 rats were given pTyr mAb, and, as expected in the lung FCM results, MASP-2-positive BM cells were at low levels of 41% and 68% in both the rats, and pTyr-positive BM cells were 53% and 29%, respectively. As shown in table 3, the values of both MASP-2-positive and pTyr-positive BM cells were high in the rats injected with MASP-2 Ab, but low in the rats injected with pTyr mAb. The Exp E rats showed lower values of BM MASP-2-positive cells (45±12%) than expected, but those of BM pTyr-positive cells were the theoretical values of 61±10%. All the Exp F rats had pTyr-positive BM cells at numbers of lower than 30%. BM cell activation of the Exp F rats was further restricted by donor cell rejection. GvH reaction in the liver, MLN and BM was aggravated by the injection of pTyr mAb. The suppression of pTyr in host cells led to donor cell activation. It was concluded that in Exp F using male hosts, female BM donor cells rejected host male-specific mHA derived from their father.

The lung electron micrographs in figure 7 show high-density granules of MASP-2 antigens stained with MASP-2 Ab-gold complexes. The type 1 alveolar cell of figure 7a shows the BM FCM results, stained with FITC-conjugated MASP-2 Ab or with FITC-conjugated pTyr mAb, in the same rats, Exp C-3, Exp E-3 and Exp F-5. In the Exp C-3 rat given MASP-2 Ab, as for the lung FCM, the BM MASP-2-positive cells were 98% and BM pTyr-positive cells were 93%. Exp E-3 and Exp F-5 rats were given pTyr mAb, and, as expected in the lung FCM results, MASP-2-positive BM cells were at low levels of 41% and 68% in both the rats, and pTyr-positive BM cells were 53% and 29%, respectively. As shown in table 3, the values of both MASP-2-positive and pTyr-positive BM cells were high in the rats injected with MASP-2 Ab, but low in the rats injected with pTyr mAb. The Exp E rats showed lower values of BM MASP-2-positive cells (45±12%) than expected, but those of BM pTyr-positive cells were the theoretical values of 61±10%. All the Exp F rats had pTyr-positive BM cells at numbers of lower than 30%. BM cell activation of the Exp F rats was further restricted by donor cell rejection. GvH reaction in the liver, MLN and BM was aggravated by the injection of pTyr mAb. The suppression of pTyr in host cells led to donor cell activation. It was concluded that in Exp F using male hosts, female BM donor cells rejected host male-specific mHA derived from their father.

The lung electron micrographs in figure 7 show high-density granules of MASP-2 antigens stained with MASP-2 Ab-gold complexes. The type 1 alveolar cell of figure 7a

**Figure 6b.** FCM of BM stained with FITC-conjugated MASP-2 Ab stage A, and FITC-conjugated pTyr mAb in stage B. A fixed border point between MASP-2-positive and -negative peaks was decided by the result of control rat Cont M3. The MASP-2-positive cell percentage was 99% (Cont M3), 98% (Exp C-3), 41% (Exp E-3) and 68% (Exp F-5) at stage A. The results of pTyr-positive cells were followed to the MASP-2 border point. pTyr-positive cell % at stage B was 90% (Cont M3), 93% (Exp C-3), 53% (Exp E-3) and 29% (Exp F-5) in the same rats shown for stage A. The Exp C-3 rat was treated with MASP-2 Ab, and the Exp E-3 and Exp F-5 rats were treated with pTyr mAb.

**Figure 7a.** Electron micrograph of a squamous alveolar cell (type I cell) derived from a control female rat (Cont F3), stained with MASP-2 Ab-gold before fixing (immunochemical stain). In the upper left part of the cytoplasm, a MASP-2-positive site is shown as a high-density round granule, with a pair of close-by centrioles. At the upper right another granule stained with MASP-2 Ab-gold is also seen. Magnification indicated at lower left (uranyl acetate-lead citrate double-stain).

**Figure 7b.** Electron micrograph of a squamous alveolar cell derived from Exp C-3 male rat, which had been given MASP-2 Ab, followed for 3 days with heparin. The lung cell suspension was stained with MASP-2 Ab-gold before fixing. Compared with control lung cells (fig. 7a), the granule of MASP-2 Ab-gold complexes was larger (i.e., activated) in the Exp C-3 rat. Two small granules positive for MASP-2 Ab-gold are also observed. To the left of the two MASP-2-positive granules, a destroying MASP-2-positive granule that must have reacted with MASP-2 Ab is present. Magnification indicated at lower left (uranyl acetate-lead citrate double-stain).

**Figure 7c.** Electron micrograph of a squamous alveolar cell is derived from Exp D-2 female rat which had been given pTyr mAb. The lung cell suspension was stained with MASP-2 Ab-gold before fixing. Compared with those of cells from control rat (Cont F3, shown in fig. 7a), two very tiny MASP-2-positive granules are seen at the upper right and under left sides of near to nucleus. The mitochondrial membranes are stained non-specifically, but more strongly, with this immunochemical staining than those of Cont F3. Magnification indicated at lower left (uranyl acetate-lead citrate double-stain).
The MASP-2 polypeptide chain has 686 amino acid (aa) residues, including a 15 aa single peptide. One mutated MASP-2 had a substitution of glycine for aspartic acid at aa 120 in the CUB1 domain found in CC1r/CC1s, Uegf and bone morphogenic protein 1. In association with the low levels of both MASP-2 and a short splice product of MASP-2 (MAP19), and severe hypocomplementemia with anti-C1q autoantibodies and low C1q levels, were detected in this mutated case. As the mutated MASP-2 failed to bind to MBL, a severe pneumococcal infection which developed was suspected to be due to MASP-2 insufficiency. In the 2000s, at least 7 types of naturally occurring polymorphisms of MASP-2 have been reported: R99Q, D120G and P126L (MAP19), and, especially, peribronchovascular edema.

DISCUSSION

The MASP-2 polypeptide chain is derived from Cont F3 rat. The type I cell of figure 7b is derived from Exp C-3 rat injected with MASP-2 Ab and the type I cell of figure 7c is derived from Exp D-2 rat injected with pTyr mAb. All the 3 cells were squamous alveolar cells without Lb. MASP-2 Ab injection did not lead to the suppression of MASP-2, while pTyr mAb injection led to suppressed expression of MASP-2, as shown in FCM. MASP-2 antigens destroyed by MASP-2 Ab were recovered quickly by activated expression of another MASP-2. When MASP-2 antigen was suppressed by pTyr mAb, mitochondrial membranes were stained non-specifically with MASP-2 Ab-gold complexes. In particular, in BM cells, MASP-2 Ab-gold complexes were actively taken up in the mitochondrial membranes, when they were attacked by pTyr mAb. In the Exp E BM cells, mitochondrial membranes must have remained relatively normal, as reflected by the low MASP-2 values (45±12%) of Exp E BM cells. It is possible that MASP-2 Ab-gold complexes did not indicate single MASP-2, even when stained positively with MASP-2 Ab. However, when a granule was stained positively with MASP-2 Ab, it is certain that the granule contained MASP-2-positive antigens to react to MASP-2 Ab.

Pulmonary surfactant protein-A (SP-A) has a similar domain structure and architecture to MBL, but lacks any intrinsic complement activity. SP-A cannot bind to MASP-2 and cannot activate complement, although MASP binding of SP-A can be easily engineered through 3 aa substitutions to the collagenous domain of MBL. The SP-A binding site in MBL and ficolins is characterized by a distinct motif within the collagenous domain: Hyp (hydroxyproline)-Gly-Lys-Xaa (aliphatic residue)-Gly-Pro. In SP-A, Glu, Cys and Glu were replaced by lysine, leucine, and proline residues, respectively, to establish new MASP binding, in which the lysine was essential for binding. Pulmonary surfactant stored in Lb is a complex mixture of lipids (phospholipids) and proteins (surfactant apoproteins), which forms a lining layer at the air-liquid interface. It has been reported that intratracheal administration of exogenous surfactant decreased intra-alveolar edema formation and the development of atelectases, and, especially, peribronchovascular edema.

Activated apocrine secretion from bronchiolar Clara cells could be explained as being initiated by mediators interacting with RTK activating phospholipase C (PLC), as mediated by G protein-coupled receptor (GPCR) signals. Increased apocrine secretion of Clara cells, as occurs in asthma, was an indirect indication of the acute lung edema in these rats injected with MASP-2 Ab.

Eosinophil granulocytes are found in the respiratory...
mucosa, the gastrointestinal tract and lymphocyte-associated organs. Elevated eosinophil cationic protein (ECP), which is the best known basic protein in eosinophil granules, is found in T helper lymphocyte type 2 diseases, such as allergic asthma and allergic rhinitis. ECP is a peptide of 133 aa with the first 40 aa necessary for membrane interfering heparin binding and cytotoxic activity. However, the mast cell products and eosinophil major basic protein (MBP) are correlated to a greater degree with bronchial hyperactivity. In this study, eosinophils were often found infiltrated in the rat lungs with pre-edematous changes of bronchial hyperactivity. Mast cell proliferation was observed in the lung edema. The intestinal LN were enlarged in the MASP-2 rats and the eosinophil granules stained positively with MASP-2 Ab. In this study, MASP-2-positive complexes of eosinophil granules were explained as follows: Firstly, rat ficolin-B in the eosinophil granules was set up for the MASP-2 binding. Rat ficolin-B corresponds to human M-ficolin, although rat ficolin-A corresponds to human L-ficolin. Recently, rat ficolin-B has been reported to have activated MASP-2 in a manner comparable to that of ficolin-A, although the activation was half that of ficolin-A. It was therefore concluded that the MASP-2 complexes in the eosinophil granules that stained positively with MASP-2 Ab must be ficolin-B-MASP-2 complexes.

In this study, lung thrombosis formation was prominent when MHC class I mAb was injected together with MASP-2 Ab. Serine protease inhibitor antithrombin III (AT-III) was judged not to be low in these MASP-2 Ab rats because serine protease activity should be high in these rats. Furthermore, MASP-2 activation, which has factor Xa-like activity and activates thrombin, must have led to fibrin clot formation in these MASP-2 rats. Heparin inhibits MASP-1 and -2 and thrombin activities together with AT-III. Heparin sodium is therefore expected to react effectively to prevent fibrin clot formation, which involves thrombosis. Although clinical hemolytic events are more apparent than thrombosis in these MASP-2 rats, fibrin clot formation in these MASP-2 rats injected with heparin, it was correct that, as a whole, small amounts of heparin sodium injection acted effectively to prevent the hypercoagulopathy induced by MASP-2 activation. Active vacuole formation of type II cells, which reflected active secretion of lung surfactant, was recognized in the MASP-2 rats injected with heparin to repair injured lung tissue. Heparin injection was absolutely necessary as an effective anticoagulant, when MHC Abs were detected in addition to MASP-2 Ab.

The rats injected with pTyr mAb, which showed suppressed expressions of MASP-2 and pTyr in FCM, exhibited a liver type of FL-Ron RTK (deleted SF-Ron PK). In the experimental rats that received pTyr mAb, splenocytes might have had a greater production of IFN-γ. Kupffer cells might have produced high levels of tumor necrosis factor alpha (TNF-α), like the Ron RTK-/- type. In this study, acute severe liver injury was not observed, but cytokine-induced moderate liver injury was seen in the rats with pTyr mAb injection, the hepatocytes of which had accelerated apoptosis with impaired Golgi apparatus. Chemotactic inhibitor, peoniflorin (PF), has been reported to have anti-inflammatory capacity against TNF-α-induced chemokine production in human dermal microvascular endothelial cells by blocking nuclear factor-kB (NF-kB) and extracellular signal-regulated kinase (ERK) 1/2 pathways. As macrophage chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), intereleukin-1 receptor antagonist (IL-1ra), IL-6, keratinocyte chemoattractant (KC), and tissue inhibitor of metalloproteinase (TIMP-1) have previously been reported to be elevated in the media from Ron RTK-/- cells, in these rats with pTyr mAb, chemotactic inhibitor was judged to have reacted effectively to suppress cytokine-induced chemokine reactions.

Judging from the results of suppressed MASP-2 and pTyr expressions of BM cells, the B and myeloid cells of the experiment rats injected with pTyr mAb might also have disturbed functions. B cell and myeloid cell development has been found to require Bruton tyrosine kinase (Btk) in a mouse model. Neutrophil migration is significantly reduced in Btk-deficient animals with enhanced granulopoiesis in the BM. Human mutation in the Bruton tyrosine kinase (BTK) gene causes X-linked agammaglobulinemia (XLA). BTK-deficient B cells lead to a complete loss of peripheral B cells. On the other hand, male-specific genes are located on the nonrecombining region of the Y chromosome. Epitopes of male-specific mHA are identified as immunogens that cause GvHD. DBY, a model of male-specific mHA revealed a high CD4-positive T cell response in a male recipient of transplanted BM cells from a human histocompatibility leukocyte antigen (HLA)-identical female sibling. After transplant, the patient developed very high reactivity to the 19-mer peptide DBY30-49 which persisted from 8 to 21 months post-transplantation. The antibody responses were mapped to 2 DBY peptides at positions 118–134 maximally at 16 months after transplant and 536–552 maximally at 21 months after transplant, which indicated that antibody response to DBY evolved by targeting a new DBY epitope. In this study, host male-specific mHA received pTyr mAb was rejected by female donor cells, in which leukocytes had impaired motility. Atypical acute GvHD due to male-specific mHA was recognized mildly or moderately in the Ron RTK deficient-like rats.
It was also suspected that activated coagulation factor XIII (FXIIIa) must have been affected in this study by reduced tyrosine kinase-dependent actions. FXIIIa works to stabilize fibrin clot and on the platelet surface plays a role in the development of highly procoagulant-coated platelets. Platelets usually bind to FXIII through αIIbβ3 integrins, which requires tyrosine kinase-dependent signals, in a potentially collaborative binding of αvβ3. In the rats treated with pTyr mAb, FXIIIa involved in a down-regulated tyrosine kinase mechanism, and instead, bound to platelets through catalytic A-subunits of FXIII (FXIII-A). Low levels of MASP-2 did not split FXIII and platelets actively. FXIIIa supported more strongly platelet activation and enhanced thrombosis formation, together with subendothelial matrix proteins, such as collagen and von Willebrand factor (VWF), under blood flow conditions. Apoptotic hepatocytes discharged into venous sinusoids blocked blood flow in these experimental rats with GvH reactions, which had an accelerating influence upon thrombosis formation.

**ΠΕΡΙΛΗΨΗ**

Πνευμονικό οίδημα και θρόμβωση που προκαλούνται από ενεργοποίηση αντιδράσεων με MBL σε συνδυασμό με MASP-2 και αντιδράσεων του MHC τάξης Ι, καθώς και ηπατική θρόμβωση προκαλούμενη από αντιδράσεις καταστολής της φωσφοτυροσινίας σε θήλεις δότες και ειδικά αντιγόνα σε άρρενες αρουραίους Lewis

**ΣΚΟΠΟΣ**

Διερεύνηση του πνευμονικού οίδηματος και της θρόμβωσης, καθώς και του ηπατικού εμβολισμού. ΥΛΟΚΑΙ ΜΕΘΟΔΟΣ Χρησιμοποιήθηκαν ποντίκια Lewis. Για την πρόκληση πνευμονικού οίδηματος χορηγήθηκε MBL (mannose-binding lectin) σε συνδυασμό με αντίσωμα MASP-2 (serine protease 2). Η θρόμβωση πνευμόνων και ήπατος, καθώς και ο εμβολιασμός, προκλήθηκαν με τη χρήση ομολογικού και αντισώματος (mAb) κατά του MCH τάξης Ι και αντισώματος κατά της MASP-2. Οι εμβολιασμοί του ήπατος ευοδώθηκαν με mAb ψωφοφοτοροσινίας (pTyr) και αντιδράσεις ειδικών αντιγόνων κατά των ελασσόνων αντιγόνων ισοσυμβατότητας (mHA). Οι σχετικές εκφράσεις της MASP-2 και της pTyr επιβεβαιώθηκαν με κυτταρομετρία ροής (FCM).

**ΑΠΟΤΕΛΕΣΜΑΤΑ**

Το αντίσωμα κατά της MASP-2 προκάλεσε οξύ ενδοκυψελιδικό και περιβρογχοαγγειακό οίδημα, με χαρακτηριστικά ταυτόχρονα αιμολυτικών ερυθροκυτταρικών κυλίνδρων κυτταρική ενεργοποίηση τύπου II με έκκριση μεμβρανικών σωμάτων (Lb) και αυξημένη αποκρινική έκκριση των κυττάρων Clara. Το MAb κατά του MHC τάξης Ι και το Ab κατά της MASP-2 προκάλεσαν βαριά θρόμβωση πνεύμονα και ήπατος, καθώς και εμβολιασμό. Οι ανοσοϊστοχημικές χρωματισμένες μικροφωτογραφίες ηλεκτρονικού μικροσκοπίου των κυψελιδικών κυττάρων τύπου I έδειξαν μια άλλη ταχεία ενεργοποίηση της MASP-2, ενώ η άλλη MASP-2 καταστράφηκε από το Ab κατά της MASP-2. Σε αυτόν τον περίπτωση είχε συμβεί εξάπλωση κυτταρών με διαταραχές της τυροσινικής κινάσης (pTyr) και αντιδράσεις ειδικών αντιγόνων κατά της pTyr mAb αύξησε την ήπατικη θρόμβωση λόγω διαταραχών της κυτταρικής κυτταρικής κυτταρικής κυττάρας (Lb). Η θρόμβωση του ήπατος που επιβαρύνθηκε από απόρριψη του θήλεος δότη από τα ειδικά mHA του άρρενος, συνδυάζοντας με διάταση των ηπατικών φλεβών. Η ντισμιοφόρος ηπαρίνη αύξησε την έκκριση πνευμονικού επιφανειοδραστικού παράγοντα (surfactant, Lb). Ο χημειοτακτικός άναπτυξιακός κατά της χημειοκίνησης, οι οποίες προαγούνταν από αυξημένη ηπατική θρόμβωση.

**ΣΥΜΠΕΡΑΣΜΑΤΑ**

Πνευμονικό οίδημα και θρόμβωση που προκαλούνται από αντιδράσεις καταστολής της φωσφοτυροσινίας σε θήλεις δότες και ειδικά αντιγόνα σε άρρενες αρουραίους Lewis

**Λέξεις ευρετηρίου:** Εμβολιασμός ήπατος, Mannose-binding lectin (MBL)-associated serine protease 2, Πνευμονική θρόμβωση, Πνευμονικό οίδημα, Τυροσινική κινάση
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