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Beneficial Effects of Perfluorotributylamine/Pluronic F-68 Stem-Emulsion (FC43se) on Discordant Lung Xenografts

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ABSTRACT

The aim of this study was to assess the effectiveness of Perfluorotributylamine/Pluronic F-68 Stem-Emulsion (FC43se) against hyperacute rejection in rabbit-pig xenodiscordant transplantation in an ex-vivo lung model. Rabbits were divided into two groups. In Group 1 (control), after extraction, the lungs were connected directly to the experimental circuit as soon as possible. The lungs were then perfused with 100 ml of pig blood only. In Group 2 (FC(+) Group), after extraction, the lungs were connected to the same circuit as soon as possible. The lungs were then perfused with 10 ml of FC43se plus 90 ml of pig blood. The duration of perfusion was defined from when the perfusion started until the PAP reached 50 mmHg. Significant differences in survival were seen between Group 1 and Group 2: in Group 1, the survival time was 51.9 ± 16.8 min, whereas in Group 2 the survival time was 76.9 ± 12.4 min ($p<0.01$). The wet-to-dry weight ratio after 30 min of perfusion in Group 2 was significantly lower than that of Group 1 and the mean pulmonary artery pressure of Group 2 at 35, 40 and 45 min after perfusion was significantly lower than that of Group 1. Histological examination revealed that FC43se suppressed the adhesion of leukocytes to the surfaces of endothelial cells, and also attenuated the intimal edematous changes accompanying leukocyte infiltration. Therefore, FC43se has a beneficial effect on suppressing hyperacute rejection in xenogeneic discordant lung transplantation.

***Key words**: Perfluorochemical, Discordant xenograft, Lung transplantation*

Perfluorocarbon was originally developed as an artificial blood substitute in 1970[5)]. Due to side effects such as the attenuation of phagocyte migration[15)], increased infection mortality and decreased neutrophil migration[16)], its clinical applications have been abandoned. However, in 1995, Wada and associates observed that Perfluorotributylamine/Pluronic F-68 Stem-Emulsion (FC43se: Green Cross, Japan), which is one of the perfluorocarbons, was effective against hyperacute rejection in the heart model of guinea pig to rat xenodiscordant transplantation[31)]. Since then, many studies in the transplantation field have focused on FC43se. However, the mechanisms responsible for FC43se's suppression of hyperacute rejection have not yet been elucidated.

As a strategy to overcome the hyperacute rejection phenomenon of xenotransplantation, there have been remarkable discoveries in the development of transgenic animals expressing species-specific complement inhibitors. In 1996, Schmoeckel and associates[27)] reported that they could prevent hyperacute rejection by using human decay accelerating factor in xenogenic, perfused working hearts. Since their report, much research in transgenic animals has been carried out. However, in 1997, Pierson and associates[23)] reported that human decay accelerating factor could not completely suppress hyperacute rejection in the lung model. They concluded that complement-independent pathogenic mechanisms might present in the lung. To date, there are no drugs that can effectively suppress hyperacute rejection in the ex-vivo lung model. Therefore, we tested the effects of FC43se, which may act through a complement-independent mechanism, against hyperacute rejection in the discordant lung xenotransplantation model.

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MATERIALS AND METHODS

Animals

Male Japanese white rabbits weighing 2.1±0.1 kg were purchased from Shimizu Jikken Inc. The rabbits were housed in the animal center for at least 1 week prior to the experiment. Male pigs (25.2±2.1 kg), which were obtained from the Faculty of Applied Biological Science at Hiroshima University, were used as blood donors. The animal received humane care in compliance with the "Principle of Laboratory Animals" prepared by the National Academy Care formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication no. 86–23 revised 1985).

Experimental Design

For anesthesia, the rabbits were given atropine sulfate (Fuso Pharm. Co., Osaka) 0.05 mg/kg and pentobarbital (Abbott Laboratories Co., U.S.A.) 50 mg/kg intramuscularly. Heparin (Leo Pharm. Prod. Co., Denmark) at 500 U/kg was then injected intravenously. A tracheotomy was performed, and a 3-mm (internal diameter) endotracheal tube was inserted. Pancuronium bromide (Organon. Techniq., the Netherlands) at 4 mg/kg was then injected intravenously. Ventilation was controlled with a Harvard artificial ventilator (type 665, volume cycled) under the following conditions: FiO2 = 1.0, Vt = 10 ml, and f = 25 /min. With the heart still beating, a 10 Fr blood-supply tube was inserted into the pulmonary trunk through the right ventricle. A 18 Fr blood-collection tube was then inserted into the left atrium through the apex. After the tubing, the endotracheal tube was fixed in place while the lungs were inflated with 10 ml/kg oxygen. Both sides of the pleura were then reflected, and the vena cava and ascending aorta were ligated. The heart and lungs were excised en bloc so as not to injure the lungs.

The heart and lungs were immediately placed in an original acrylic box (20×20×30 cm), with the interior humidity maintained at 100%. The box was placed in an incubator (Thermo. minder SM-05: TAITEC Co., Tokyo) with the temperature set at 38°C. During the above procedures, except for the administration of heparin to prevent thrombosis, neither parenteral fluids nor chemicals were used. After the extraction of the lungs, no further treatments were performed.

Experimental Groups

The rabbits were divided into two groups. Each group was then further subdivided into two more groups: Group A, in which the lungs were perfused for as long as possible, and Group B, in which the lungs were perfused for 30 min exactly and then subjected to histological and biochemical analysis.

In Group 1 (control; n(A)=7; n(B)=6), after extraction, the lungs were connected directly to the experimental circuit as soon as possible. The lungs were then perfused with 100 ml of pig blood with 0.4 g of trisodium citrate, dihydrate (Wako Pure Chemical industries LTD., Osaka). In Group 2 (FC(+) Group; n(A)=7; n(B)=6), after extraction, the lungs were connected directly to the experimental circuit as soon as possible. The lungs were then perfused with 10 ml of FC43se plus 90 ml of pig blood with 0.4 g of trisodium citrate, dihydrate. The FC43se was mixed in solution when the blood-supply tube was inserted, because the blood flow to the lungs was thought to be blocked by the roller pump (JMS; infuser Model M-04, Hiroshima). The duration of the ischemic state was 10 min in both groups. The closed circuit is indicated in Fig. 1. The circuit consisted of a tube (No.23: JMS, Hiroshima; internal diameter; 6.4 mm) filled with lactated Ringer's solution (SOLULACT®: TERUMO, Tokyo). Before perfusion, the lung was flushed out with 100 ml of lactated Ringer's solution for 5 min. Blood maintained in a reservoir at 38°C was delivered to a membrane oxygenator (Model EC-30: Senko Ika Co., Tokyo) with a roller pump. Blood that had passed through the ventilated lungs (atrial blood) was collected from the left atrium into another reservoir. The flow speed was set at 4 ml/kg/min at first, but increased at 4 ml/kg/min per min until a constant 20 ml/kg/min was reached. An air-mixer (SECHRIST® model 3500 HL; Japan Life Line Co., Tokyo) was connected to a membrane oxygenator, and was set to supply 1 liter of mixed gas (N_2 : CO_2 = 9 : 1) under the following conditions: PvO_2=60 mmHg

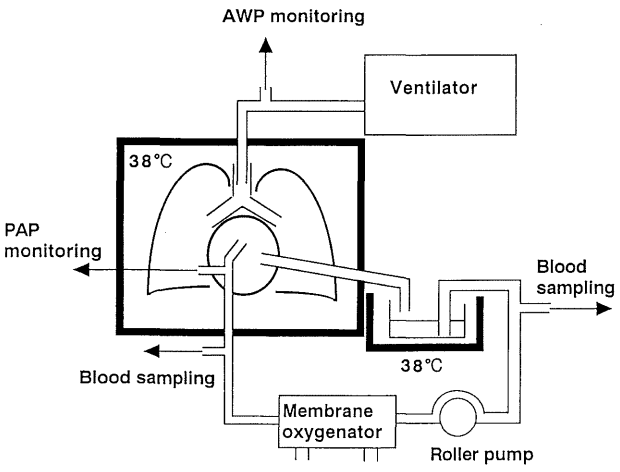

Fig. 1. The perfusion circuit.
AWP, airway pressure: PAP, pulmonary artery pressuer.

and $PvCO_2$=50 mmHg at 10 min postperfusion. In the above procedure, at 20 min after perfusion, the blood coming from the membrane oxygenator was stable venous blood (PvO_2=56.8±4.2 mmHg, $PvCO_2$=51.2±3.1 mmHg). The extracted lungs were ventilated with the Harvard artificial ventilator under the following conditions: FiO_2=1.0, Vf= 10 ml/kg; f= 25 /min; and PEEP=2.0c mH_2O.

Data Collection

The lungs in Group A were perfused continuously until the mean pulmonary artery pressure (PAP) reached 50 mmHg, after which perfusion was determined to be impossible. The duration of the perfusion (perfusion time) was measured from when the perfusion started until the PAP reached 50 mmHg. During the perfusion, both the PAP and the maximum airway pressure (AWP) were measured continuously. One milliliter of blood was sampled from the left atrium every 10 min in order to measure the PaO_2 and $PaCO_2$. The PAPs and AWPs were measured with a pressure transducer, and were recorded continuously with a polygraph (360 system: San-ei, Tokyo). The lungs in Group B had their perfusion stopped at exactly 30 min. The blood was sampled for the biochemical analysis of immunoglobulin M (IgM), complement levels (CH50), lactic dehydrogenase (LDH) and for hematological study at 5 and 30 min after the perfusion started.

The platelet and white blood corpuscle (WBC) counts were performed automatically by a Celltac α Counter (Nihon Kohden, Japan). The LDH was measured automatically by a HITACHI 7170 (Hitachi, Japan) using LICHITEC® LDL opt. (Behringer Manheim, Germany). The CH50 was measured by the micro titer method according to Mayer[20]. The IgM titer was measured by the general radial immunodiffusion (RID) method[19] using a Binding Site RID kit (The Binding Site Limited, England).

The upper lobe of the right lung was fixed immediately after the cessation of perfusion with 10% formaldehyde for histological analysis. The sample was sectioned, stained with hematoxylin and eosin, and then observed by light microscopy. The upper lobe of the left lung was used to measure the wet weight. The left lungs were dried in a dessicator at 80°C for 48 hours, and the dry weight was then measured. The wet-to-dry weight ratio (W/D) was also calculated.

Differences in the perfusion time, W/D and biochemical parameters were compared with the student's t-test. The PaO_2, $PaCO_2$, PAP and AWP were compared with the Wilcoxon rank-sum test. Differences were considered to be statistically significant if the p values were less than 0.05. The results are represented as means±SEM.

RESULTS

Perfusion Time

FC43se effectively prolonged the survival of the discordant xenograft. A total of 14 lungs were eligible for this study. Significant differences in the survival time were seen between Group 1 and Group 2. In Group 1, the survival time was 51.9±16.8 min, whereas in Group 2 the survival time was 76.9±12.4 min ($p<0.01$) (Fig. 2).

PaO_2

The FC(+) lungs displayed significantly better oxygenation than the control lungs after 20 min, 30 min and 40 min of perfusion. After 20 min of perfusion, the control lungs had a PaO_2 of 483±23 mmHg; the FC(+) lungs had a PaO_2 of 513±27 mmHg ($p<0.05$). After 30 min of perfusion, the control lungs had a PaO_2 of 448±32 mmHg versus 498±30 mmHg for the FC(+) lungs ($p<0.05$). After 40 min of perfusion, the control lungs had a PaO_2 of 418±42 mmHg versus the FC(+) lungs with 513±20 mmHg ($p<0.01$).

$PaCO_2$

Even after 10 or 20 min of perfusion, no significant differences were noted in the $PaCO_2$ values between the control and FC(+) groups. After 30 min of perfusion, the control lungs had a $PaCO_2$ of 43±3 mmHg whereas the FC(+) lungs had a $PaCO_2$ of 37±4 mmHg ($p<0.05$) (Fig. 3).

Mean Pulmonary Artery Pressure (PAP)

No significant differences were observed during 5 min to 25 min of perfusion in the PAP. However, the PAP of the control group became significantly higher than the FC(+) group. The PAPs were as follows: at 30 min, control 28±5 mmHg and FC(+) 20±3 mmHg; at 35 min, control 29±4 mmHg and FC(+) 22±3 mmHg; at 40 min, control 27±2 mmHg and FC(+) 22±4 mmHg; and at 45 min, control 31±6 mmHg and FC(+) 23±4 mmHg.

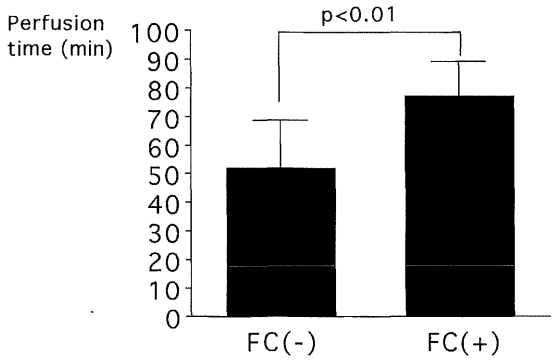

Fig. 2. Duration of the perfusion time from when the perfusion was initiated until the pulmonary artery pressure reached 50 mmHg.

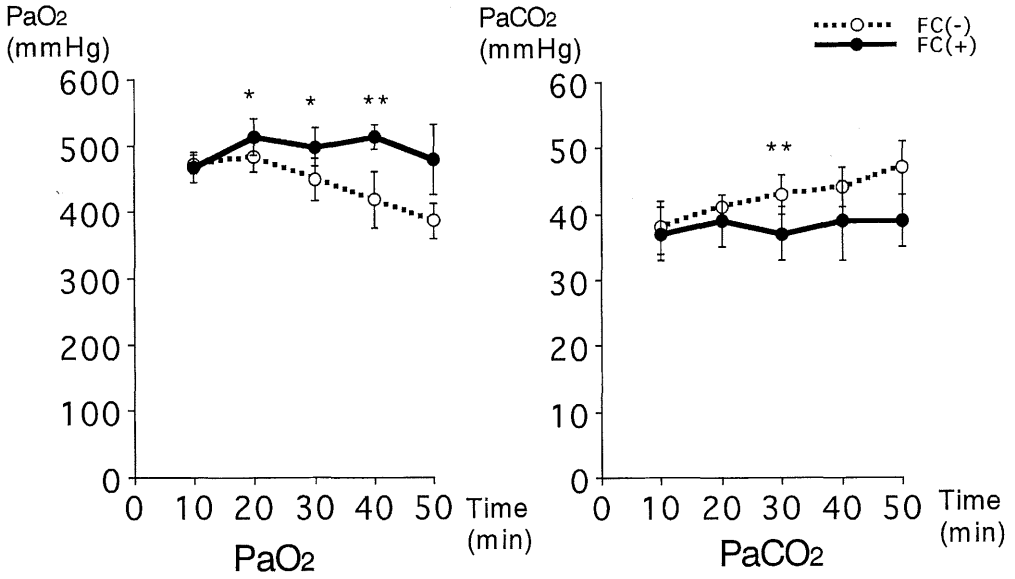

Fig. 3. The PaO_2 and $PaCO_2$ of blood sampled from the left atrium.
*p<0.05, **p<0.01

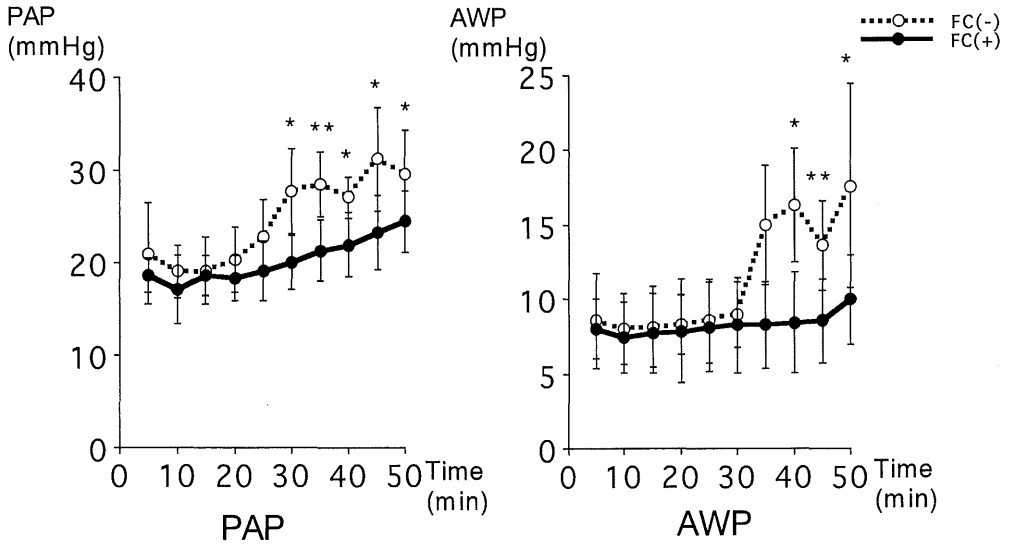

Fig. 4. Pulmonary artery pressure (PAP) and airway pressure (AWP) during perfusion.
*p<0.05, **p<0.01

Maximum Airway Pressure (AWP)

The AWP after 5 min of perfusion was: control 9±3 mmHg versus FC(+) 8±2 mmHg. No significant differences were observed. However, the subsequent AWPs were as follows: at 35 min, control 15±4 mmHg and FC(+) 8±3 mmHg; at 40 min, control 16±4 mmHg and FC(+) 8±3 mmHg; and at 45 min, control 14±3 mmHg and FC(+) 9±3 mmHg. The AWP of the control group became significantly higher than the FC(+) group (Fig. 4).

Wet/Dry Weight Ratio (W/D)

The W/D after 30 min of perfusion was: control 5.44±0.14 versus FC(+) 5.10±0.14. The W/D of the FC(+) group was significantly lower than the control group (p<0.01) (Fig. 5).

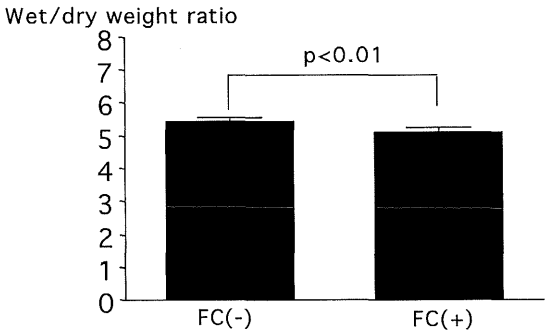

Fig. 5. The wet/dry weight ratio after 30 min of perfusion.

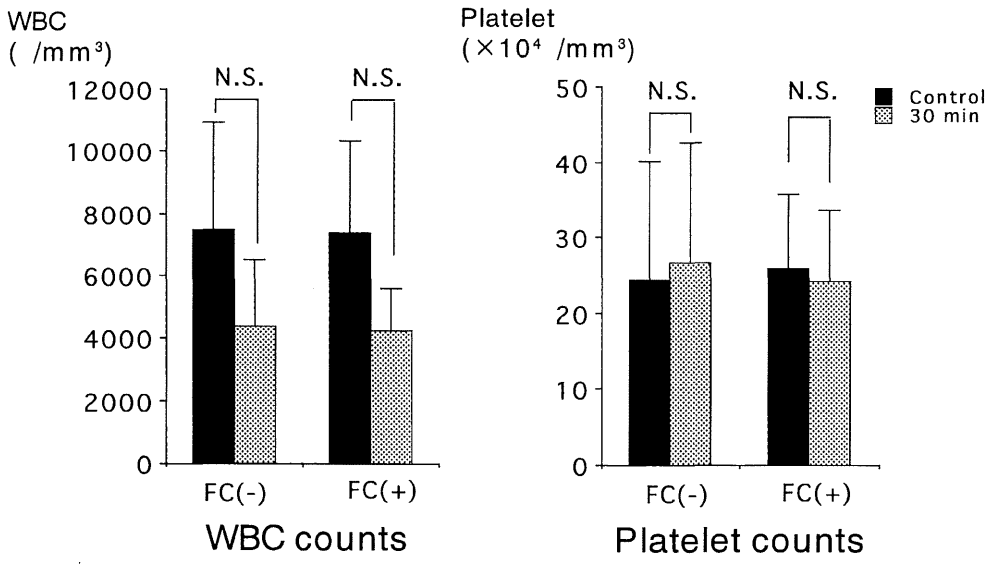

Fig. 6. WBC and Platelet counts.
There were no significant differences.

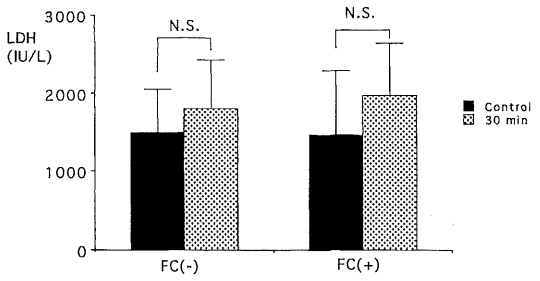

Fig. 7. Changes in the LDH levels.
There were no significant differences.

Hematological and Biochemical Analysis

(a) WBC

At the start of the perfusion, the WBC counts of the control and FC(+) groups were as follows: control 7456±3464 /ml versus FC(+) 7401±2936 /ml. The WBC counts after 30 min of perfusion were as follows: control 4382±2106 /ml versus FC(+) 4251±1324 /ml. There were no significant differences.

(b) PLT

At the start of the perfusion, the PLT values of the control and FC(+) groups were as follows: control $24.3 \pm 15.8 \times 10^4$ /ml versus FC(+) $25.8 \pm 9.9 \times 10^4$ /ml. The PLT values after 30 min of perfusion were as follows: control $26.7 \pm 15.9 \times 10^4$ /ml versus FC(+) $24.1 \pm 9.5 \times 10^4$ /ml. There were no significant differences (Fig. 6).

(c) LDH

At the start of the perfusion, the LDH levels of the control and FC(+) groups were as follows: control 1496±560 IU/liter versus FC(+) 1470±820 IU/liter. The LDH levels after 30 min of perfusion were as follows: control 1800±622 IU/liter versus FC(+) 1972±663 IU/liter. There were no significant differences (Fig. 7).

(d) CH50

At the start of the perfusion, the CH50 values of the control and FC(+) groups were almost equal. The CH50 values after 30 min of perfusion were as follows: control 15.5±1.6 CH50 U/ml versus FC(+) 14.5±0.8 CH50 U/ml. There were no significant differences.

(e) IgM

At the start of the perfusion, the IgM levels of the control and FC(+) groups were almost equal. The IgM levels after 30 min of perfusion were as follows: control 130.7±53.6 mg/dl versus FC(+) 116.2±41.4 mg/dl. There were no significant differences (Fig. 8).

Histological Examination

In the FC(–) grafts, a number of leukocytes adhered to the surfaces of the endothelial cells. Edematous changes in the intima accompanied by leukocyte infiltration were also recognized in the arterial and venous walls. Leukocytic thrombi were observed in the vessels of all cases (Fig. 9a,b,c). On the other hand, the findings described above were difficult to observe in the FC(+) grafts (Fig. 9d).

DISCUSSION

According to the International Lung Transplant Registry, the 1-year and 2-year survival rates of

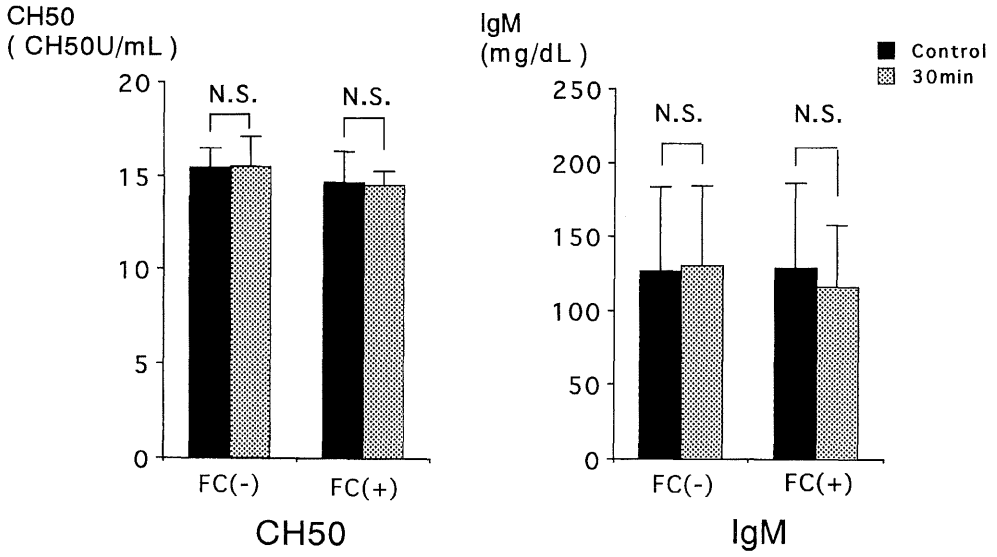

Fig. 8. Changes in the CH50 values and in the IgM titre. There were no significant differences.

lung transplantation are 70% and 60%, respectively. Since the first successful lung transplantation in 1963[8], more than 5000 transplantations have been performed, and lung transplantation is currently an accepted treatment for patients with end-stage pulmonary disease. However, the number of lung transplantations seems to be reaching a plateau; the numbers were 1033 in 1993, 1098 in 1994, 1219 in 1995 and 998 in 1996[9,10]. The most direct cause of this plateau is thought to be the short supply of human organs. Therefore, studies on xenogenic discordant lung transplantation are necessary to compensate for the shortage of human organs.

In discordant lung transplantation, hyperacute rejection, which is a phenomenon characterized by the development of edema, loss of blood flow and the subsequent deterioration of gas exchange[2], is the most difficult problem to overcome. However, there is a general consensus that complement activation is an essential step in the development of hyperacute rejection[18,25], although the mechanisms causing these processes have not yet been elucidated[1,24]. The most urgent problem to address is how to control hyperacute rejection effectively. In recent years, various methods such as the use of specific immunoadsorbent columns[17], the injection of hapten antigens to block preformed natural antibodies[6], and the cultivation of transgenic animals[27] have been proposed. However, these studies are not ready for clinical application. In transgenic animals, hyperacute rejection was prevented by human complement-regulatory proteins, in the heart model[27], but was not always prevented in the lung model[3,23]. For the time being, there are no effective methods to suppress hyperacute rejection in lung xenotransplantation.

Perfluorochemicals are blood substitutes with a high oxygen-carrying capacity, low viscosity, and are the capability of dissolving both oxygen and carbon dioxide[5,21]. Although their biological properties have not been completely elucidated, many investigators have abandoned the idea of using these substances as clinical materials. However, Wada and associates noted FC43se's immunosuppressive effects, and demonstrated that FC43se was effective in suppressing hyperacute rejection in the guinea pig to rat xenodiscordant transplantation model[30,31]. Ueno, who was one of his associates, further showed that FC43se was also effective against allotransplantation in an ex-vivo model with rabbit lung[29]. Therefore, we hypothesized that FC43se may have a suppressive effect against hyperacute rejection in lung grafts.

Advances in cross-species transplantation of the heart, kidney, and liver suggested similar potential for pulmonary xenotransplantation, but, unlike these other solid organs, the experience with experimental lung xenotransplantation has been limited[28]. In experimental lung xenotransplantation, it is difficult to transplant orthotopically, because rejection of the grafts is too rapid to evaluate. It is also difficult to transplant heterotopically because of its anatomical structure. Therefore, experimental lung xenotransplantation was performed in an ex-vivo model[4,7], which is a similar condition to transplant in-vivo. We chose a combination of rabbit-to-pig, which was a bigger size than the combination of guinea-pig and rat, because we could monitor and record accurately. Finally, we tested whether FC43se might have a suppressive effect against hyperacute re-

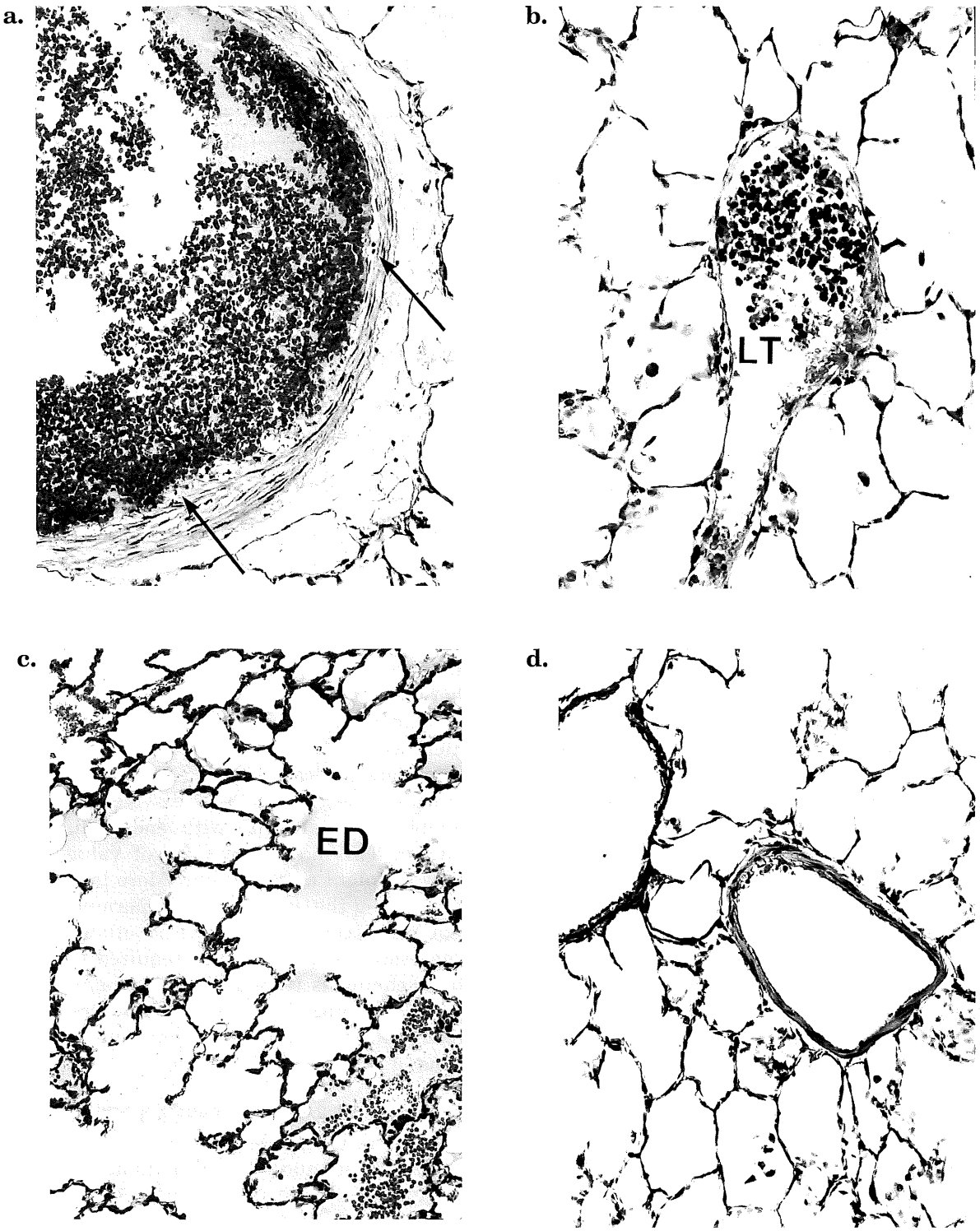

Fig. 9. Light microscopic findings of the grafted lung without FC (a-c) and with FC administration (d). Adhesion of leukocytes on the endothelial cells, intimal edema with leukocyte infiltration (a: arrows), leukocytic thrombi (b: LT) and lung edema (c: ED) were observed in the lung without FC. On the other hand, these changes were difficult to recognize in the lung with FC (d). HE stain. a, ×200, b, ×400, c, ×200, d, ×200

jection in a rabbit-to-pig discordant lung xenotransplantation model[12,14,22]. We chose a concentration of FC43se of 10%, because this density was shown to be the most effective in our preliminary studies.

In our study, the survival time of the FC43se (+) animals was one and half times as long as the survival time of the controls. The W/D ratio also showed a significant difference between the FC(+) and FC(–) groups. These results strongly suggest that FC43se has a suppressive effect on hyperacute rejection in the rabbit-to-pig ex-vivo lung

model. As far as we know, this report is significant in that it demonstrates for the first time the effectiveness of FC43se in discordant lung transplantation.

From a pathological point of view, the grafts in the control group showed leukocytes adhering to the endothelial cells and infiltrating into the intimal as well as subendothelial edema, which are characteristic of the findings observed in hyperacute rejection[2,11,14]. However, in the FC(+) group, these findings were milder. Therefore, we believe that our pathological findings demonstrate that FC43se has a suppressive effect against hyperacute rejection by reducing leukocyte adherence to the endothelial cells.

In the specimen sacrificed at 30 min, intimal edema was noted. The respiratory physiological data, such as the PaO_2, $PaCO_2$, APW and PAP, became significantly different at 30 min after perfusion. This lung edema is evidence of significant differences in the PaO_2, $PaCO_2$, AWP and PAP. Once interstitial edema occurs, the permeability of the alveoli decreases, and finally the PaO_2 becomes decreased while the $PaCO_2$ becomes increased. In accordance with the increasing compliance of the lung, the AWP and PAP values also increased. The edematous changes of the intima were reflected by the mean lung edema, which was the difference in the W/D ratio. A comparison of the W/D ratios also showed that the progress of pulmonary edema was suppressed by FC43se. This means that FC43se also inhibited the increase in the permeability of the vessels.

Some of the results we obtained were unexpected. First, the survival time was shorter than we had expected; in Wada's study[31], the FC(+) group showed 70 times the survival time of the control group. Second, the biochemical results did not show any significant differences between the FC(–) and FC(+) groups. Generally speaking, in hyperacute rejection, a complement is activated via the alternative pathway[26] and serous IgM and CH50 levels are reduced[13]. However, our data showed different results. We believe that there may be another mechanism responsible for hyperacute rejection, and that FC43se may act on this second mechanism which we could measure by different parameters.

The WBC, PLT and LDH values were measured as an index of inflammation, immediate agglutination and tissue injury. We thought that invasion into the vessels and the agglutination of WBC and PLT would be suppressed by FC43se, and the hemolysis of WBC and PLT also suppressed. Therefore, we expected a decrease in the WBC and PLT, and an increase in the LDH in the FC(+) group. Again, however, there were no significant differences. This probably means that the 30 min of perfusion in our model is not long enough for significant tissue injury to occur. If we had examined these indices later on, we may have obtained different results.

The lung is a unique organ, and has a number of different functions such as respiration, the composition of surfactant, the production of hormone and immunological modification. We think that the unexpected data could be partially attributable to the unique properties of the lung. Generally speaking, transplantations of the kidney and cornea have better results than the liver and small intestine. Similarly, we believe that the lung is one of the most sensitive organs to rejection reactions against transplantation. For example, Pierson[9] has suggested that in contrast to the heart, complement-independent mechanisms may be of critical importance to hyperacute rejection in the lung.

The combination of recipient and donor species might also be an important factor. Whether a combination is concordant or discordant is determined by the prolongation of the xenograft survival. We attempted to examine the combination of guinea pig-to-rat in our study, which was the same as in Wada's study. However, these animals were too small to record various parameters. Therefore, we used the combination of rabbit-to-pig. Our differing results must be partially due to differences in the combination.

Our data indicate that FC43se suppressed hyperacute rejection in the ex-vivo lung model, possibly by interfering with endothelial cell activation. This datum may be of value in other xenotransplant combinations. More investigations into varying the administration parameters (dose, frequency, combination with other immunosuppressive substances) are required to elucidate the mechanisms responsible for hyperacute rejection in the lung. We think that our results may provide an important key to elucidate the mechanisms of hyperacute rejection.

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