Acta Medica Okayama

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Sadanori Fuchimoto[*]

[*]Okayama University,

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Abstract

Active enhancement was induced in inbred rats with cardiac allografts using semisoluble antigens. The optimal time of antigen pretreatment and optimal dose of semisoluble antigens were examined. The presence of serum blocking factors in the sera of rats having had allografts for a long time was examined with a macrophage migration inhibition test and lymphocyte microcytotoxicity assay. Since the blocking factors of macrophage migration inhibition were increasing on the 7th day, that day was determined to be the optimal time of antigen pretreatment. The mean survival time (MST) of cardiac allografts in untreated rats was 17.2 +/- 7.5 days. Semisoluble antigens were administered at 2 mg/kg body weight 7 days before the graft, 4 mg/kg 7 days before the graft and 2 mg/kg divided over three days, 15, 8 and 1 day before the graft, and the MSTs of cardiac allografts of rats receiving these treatments were 71.2 +/- 39.9, 62.6 +/- 42.2 and 79.3 +/- 31.0 days, respectively. The MST in each group of the treated rats was significantly longer than that of the control group (p less than 0.01). Rejection of the allograft, however, was accelerated in a group treated with 8 mg/kg 7 days before the graft (MST: 8.4 +/- 3.2 days). Serum blocking factors were detected in the sera of approximately half of the rats having cardiac allografts which survived a long time.

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*PMID: 6198870 [PubMed - indexed for MEDLINE]

ACTIVE ENHANCEMENT OF RAT CARDIAC ALLOGRAFTS INDUCED BY DONOR SPECIFIC SEMISOLUBLE ANTIGENS

Sadanori Fuchimoto

First Department of Surgery, Okayama University Medical School, Okayama 700, Japan
(Director : Prof. K. Orita)

Received April 5, 1983

Abstract. Active enhancement was induced in inbred rats with cardiac allografts using semisoluble antigens. The optimal time of antigen pretreatment and optimal dose of semisoluble antigens were examined. The presence of serum blocking factors in the sera of rats having had allografts for a long time was examined with a macrophage migration inhibition test and lymphocyte microcytotoxicity assay. Since the blocking factors of macrophage migration inhibition were increasing on the 7 th day, that day was determined to be the optimal time of antigen pretreatment. The mean survival time (MST) of cardiac allografts in untreated rats was 17.2 ± 7.5 days. Semisoluble antigens were administered at 2 mg/kg body weight 7 days before the graft, 4 mg/kg 7 days before the graft and 2 mg/kg divided over three days, 15, 8 and 1 day before the graft, and the MSTs of cardiac allografts of rats receiving these treatments were 71.2 ± 39.9, 62.6 ± 42.2 and 79.3 ± 31.0 days, respectively. The MST in each group of the treated rats was significantly longer than that of the control group ($p < 0.01$). Rejection of the allograft, however, was accelerated in a group treated with 8 mg/kg 7 days before the graft (MST : 8.4 ± 3.2 days). Serum blocking factors were detected in the sera of approximately half of the rats having cardiac allografts which survived a long time.

Key words : active enhancement, optimal time of antigen pretreatment, serum blocking factor, rat cardiac allograft.

Organ transplantation, especially of the kidney, is now an accepted method in medical care. In spite of careful matching to obtain the most suitable combination between donors and recipients, prolonged use of immunosuppressive drugs is necessary. Such therapy, however, may cause serious and even lethal complications. Induction of specific immunological enhancement to the donor antigens with full reactivity against other antigens would be ideal for organ transplantation. It is said that the following factors are involved in the induction of immunological

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Abbreviations : MIT, macrophage migration inhibition test ; MI blocking activity, blocking activity of the macrophage migration inhibition ; LMCA, lymphocyte microcytotoxicity assay ; MST, mean survival time ; SA, semisoluble antigen ; LC blocking activity, blocking activity of lymphocyte cytotoxicity ; MIF, macrophage migration inhibitory factor.

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enhancement : the route of antigen administration, the initial and total dose of antigen, the duration of pretreatment, the interval between cessation of treatment and organ transplantation, the number of antigens contained in the pretreated materials, the levels and types of antibodies produced in the host and the genetic disparity between donors and recipients.

In this study active enhancement was induced by advance treatment with semisoluble antigens (SA) in inbred rats with cardiac allografts. The optimal time of antigen pretreatment and optimal dose of SA were determined. The presence of serum blocking factors in the sera of rats with long-surviving cardiac allografts was also examined by a macrophage migration inhibition test (MIT) and lymphocyte microcytotoxicity assay (LMCA) (1).

MATERIALS AND METHODS

Animals. Male inbred rats from closed laboratory populations were used throughout this study. Wistar rats weighing 200-300 g were used as recipients and MP rats of comparable weight as donors. The mean survival time (MST) of skin grafts was 7.6 ± 1.8 days in this combination which was considered to be an R antigen compatible and non-R antigen incompatible pair (unpublished data).

Cardiac allotransplantation. Cardiac transplantation was conducted using a stereomicroscope (Nikon ; × 8) under ether anesthesia according to the method of Ono *et al.* (2).

Preparation of donor heart. The donor rats underwent thoracotomy after general heparinization by injecting 1000 U/rat of heparin into the tail vein. After ligation and resection of the superior and inferior vena cava, the ascending aorta and the main pulmonary artery were excised, leaving them extending 3 mm from the radices. Then the pulmonary vein and the left atrium were excised *en masse* after ligation. After removal of the donor heart from the thorax, the heart was kept in saline with 1 % heparin at 4 ℃.

Cardiac transplantation. The recipient rats underwent laparotomy by median incision. The abdominal aorta and the inferior vena cava were exposed below the height of the renal artery and vein, respectively. Then 2-3 mm longitudinal incisions were made on them after applying bulldog clamps to each of them directory below the renal vessels and directly above the bifurcation of the abdominal aorta. The aorta and pulmonary artery of the donor heart were sutured to the abdominal aorta and the inferior vena cava of the recipient with 8-0 and 7-0 nylon gut (*Eticon* ®), respectively. In declamping, an effort was made to minimize bleeding by applying sterile, absorbable oxidized cellulose (*Oxycell* ®, cotton type) to the aortic suture lines.

Preparation of semisoluble antigen. Semisoluble antigens were prepared by a modification of the method of Kim *et al.* (3) (Fig. 1). The donor spleen was minced, homogenized and filtered through an 80-mesh filter. After excluding the red cells, spleen cells, suspended at 5×10^7 cells/ml in saline, were disintegrated ultrasonically and centrifuged. The supernatant was distributed in 1 ml ampules, stored at -20 ℃ and used as the semisoluble antigens after thawing. The mean protein concentration of the semisoluble antigen preparation used in this study was 0.53 ± 0.08 mg/ml as measured by Lowry's method (4).

Determination of the optimal time of antigen pretreatment. Recipient rats were pretreated by intravenous injection of semisoluble antigens (2 mg/kg). Sera were collected from the tail vein of the rats 1, 3, 5, 7, 9, 11, 13, 20 and 27 days after the pretreatment. The sera were
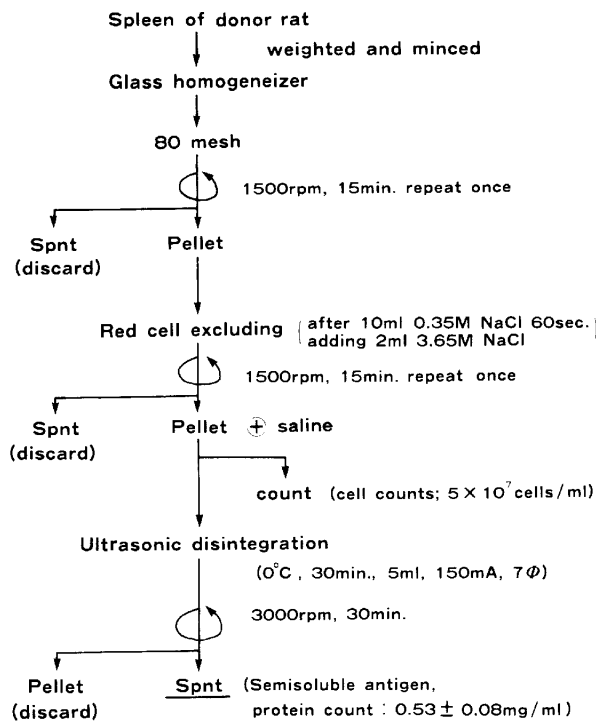
Spleen of donor rat
↓   weighted and minced
Glass homogeneizer
↓
80 mesh
↻ 1500rpm, 15min. repeat once

Spnt   Pellet
(discard)
↓
Red cell excluding ⎰ after 10ml 0.35M NaCl 60sec. ⎱
                   ⎱ adding 2ml 3.65M NaCl   ⎰
↻ 1500rpm, 15min. repeat once

Spnt   Pellet ⊕ saline
(discard)
↓
count (cell counts; 5×10^7 cells/ml)

Ultrasonic disintegration
($0°C$, 30min., 5ml, 150mA, 7Φ)
↻ 3000rpm, 30min.

Pellet   Spnt (Semisoluble antigen,
(discard)   protein count : 0.53 ± 0.08mg/ml)

Fig. 1. Flow scheme for preparation of semisoluble antigens

inactivated at $56°C$ for 30 min and then kept at $-20°C$. Control sera were collected from untreated recipient rats. Peripheral blood lymphocytes were isolated by the Ficoll-Conray gradient separation technique from the recipient rats 5 days after skin grafts by the method of Billingham et al. (5). The lymphocytes showed significant macrophage migration inhibition activity in the presence of semisoluble antigens (unpublished data). The MI blocking activity of test and control sera were examined by conducting a direct MIT using the lymphocytes and semisoluble antigens in the presence of test or control sera. The optimal time of antigen pretreatment was determined by marking the change in the MI blocking activity after the antigen pretreatment.

Experimental groups. Experimental rats were divided into five groups according to the time, dosage, and frequency of the administration of SA : 1) a control group, 2) a group given 2 mg/kg of SA 7 days before, 3) a group given 4 mg/kg 7 days before, 4) a group given 8 mg/kg 7 days before and 5) a group given a total of 2 mg/kg in three injections 15, 8 and 1 day before the cardiac allograft. The rats in each group underwent cardiac transplantation and the rate of rejection was followed.

Blocking factors in the sera from rats with long-surviving cardiac allografts. The presence of blocking factors was examined in heat-inactivated ($56°C$ for 30 min) sera collected from the tail vein of rats whose cardiac allografts had survived a long time.

Direct MIT. Macrophages in peritoneal exudate of guinea pigs were used as indicator cells

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by a capillary tube method (6). Control sera or test sera were added to TC-199 culture medium at a final concentration of 12.5 %, which was found to be the optimal concentration enabling the removal of non-specific macrophage sitmulation or inhibition by control sera. Sensitized lymphocytes were collected from the peripheral blood in rats with long-surviving cardiac allografts by the Ficoll-Conray gradient separation technique. Peripheral blood lymphocytes of the donor were used as antigens by disintegrating them ultrasonically. An antigen concentration of 30γ/ml was optimal for enabling the removal of non-specific macrophage migration inhibition by antigens in this experiment. The direct MIT was conducted by using these components. The MI blocking activity was calculated from the results of three replications as follows :

$$\text{MI blocking activity } (\%) = (\frac{\text{MI index with test serum}}{\text{MI index with normal serum}} - 1) \times 100 \quad , \text{where}$$

$$\text{The MI index } (\%) = (\frac{\text{the average migration area with antigens}}{\text{the average migration area without antigens}}) \times 100$$

LMCA. The LMCA was conducted according to the method of Takasugi and Klein (7). Donor kidney cells, primarily cultured by the Madden and Burk method (8), were used as target cells, and peripheral lymphocytes from rats with long-surviving cardiac allografts were used as effector cells.

Ten μl of a 1.5×10^4 cells/ml suspension of primarily cultured kidney cells was dispensed in each well of a microtest plate (Falcon Plastics Co., Oxnard, CA, USA) and incubated at 37 ℃ under a humidified 5 % CO_2-95 % air atmosphere for 24 h. After incubation, the medium was removed by aspiration, and effector cells suspended at 2.25×10^6 cells/ml in RPMI-1640 medium with 16 % control serum or immune serum were added to each well (10 μl). The cells were incubated further at 37 ℃ in an atmosphere of 5 % CO_2 for 90 min. After removal of the medium, the cells were suspended in RPMI-1640 medium with 20 % FCS, and incubated again at 37 ℃ in an atmosphere of 5 % CO_2 for 48 h. The microtest plate was inverted and shaken and the dead cells were removed by washing. The viable cells were incubated for an additional 1 h, fixed with 1.25 % glutalaldehyde and stained with crystal violet. The number of the kidney cells adhering to the microtest plate was counted under a microscope ($\times$ 100). The blocking activity of lymphocyte cytotoxicity (LC blocking activity) was calculated from the results of at least three replications as follows :

$$\text{LC blocking activity } (\%) = (1 - \frac{\text{cytotoxic index with test serum}}{\text{cytotoxic index with normal serum}}) \times 100 \quad , \text{where}$$

$$\text{The cytotoxic index } (\%) = (\frac{\text{the average number of kidney cells with effector cells}}{\text{the average number of kidney cells without effector cells}}) \times 100.$$

Statistical analysis. Comparison between the groups were analysed by Student's *t*-test. The difference between two groups was considered significant if the 'p' value was less than 0.05.

RESULTS

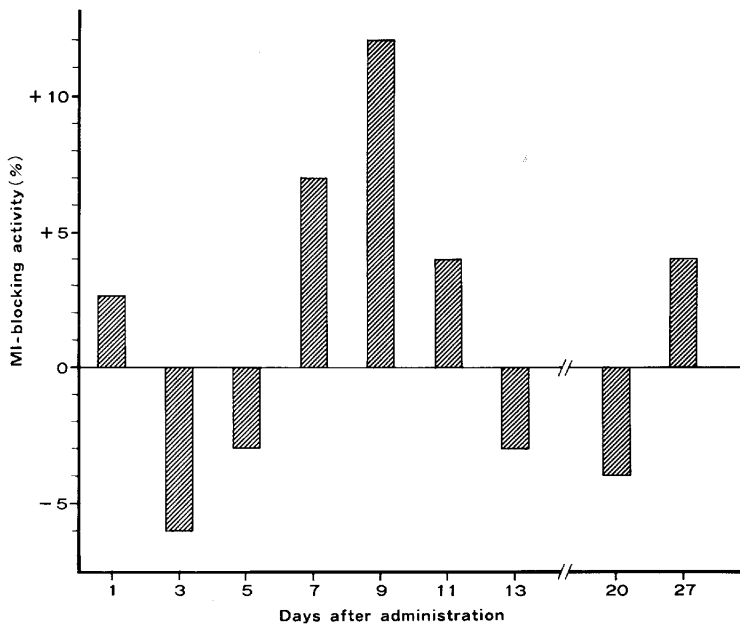*Optimal time of antigen pretreatment.* The MI blocking activities were + 2.6 %,

Fig. 2.   Daily change of MI blocking activity after administration of semisoluble antigen (2 mg/kg)

$+7.0$ %, $+12.0$ %, $+4.0$ % and $+4.0$ % one, 7, 9, 11 and 27 days after the antigen pretreatment, respectively (Fig. 2).   Since the MI blocking activity was increasing on the 7 th day, antigen pretreatment 7 days before the cardiac allograft was thought to be optimal.

Survival time of cardiac allografts.   As shown in Table 1, the MST of cardiac allografts in the untreated rats was 17.2 ± 7.5 days, and in 8-30 days all cardiac allografts ceased to function.   The MST in the rats given 2 mg SA/kg was 71.9 ± 39.9 days, and the cardiac allografts of 6 rats among the 9, in which the MST was significantly prolonged compared with the untreated rats ($p < 0.01$), survived more than 100 days.   Likewise, the MST in the rats given 4 mg SA/kg was 62.6 ± 42.2 days, and the cardiac allografts of 5 rats among the 9, in which the MST was significantly prolonged compared with the untreated rats ($p < 0.01$), survived more than 100 days.   On the other hand, the heart ceased within 4-13 days in all the rats given 8 mg SA/kg, in which the MST of the cardiac allografts was 8.4 ± 3.2 days.   The cessation of the heart was significantly earlier than that of the untreated rats ($p < 0.01$).   In rats given 2 mg SA/kg over three days, the cardiac allografts survived more than 100 days in 4 rats among the 6, and the MST was 79.3 ± 31.0 days.   This was a significant prolongation of the MST compared with that of the untreated rats ($p < 0.01$).   There was no significant difference in the MSTs when the rats of this group were compared with those given 2 mg/kg 7 days

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TABLE 1. SURVIVAL TIME OF MP CARDIAC ALLOGRAFTS IN WISTAR RECIPIENTS

Group no.	Pretreatment	No. of rats	Individual survival times (days)	MST±SD[a] (days)	p
1	No treatment	9	8, 10, 11, 11, 16, 20, 22, 27, 30	17.2±7.5	—
2	Semisoluble antigen 2mg/kg 7 days before	9	10, 13, 25, >100, >100 >100, >100, >100, >100	71.9±39.9	[b]p<0.01
3	Semisoluble antigen 4mg/kg 7 days before	9	6, 14, 14, 29, >100, >100 >100, >100, >100	62.6±42.2	[b]p<0.01
4	Semisoluble antigen 8mg/kg 7 days before	9	4, 7, 7, 11, 13	8.4±3.2	[b]p<0.01
5	Semisoluble antigen, total 2mg/kg 15, 8, 1 days before	6	20, 56, >100, >100 >100, >100	79.3±31.0	[b]p<0.01 [c]p>0.05

a Mean survival time± standard deviation
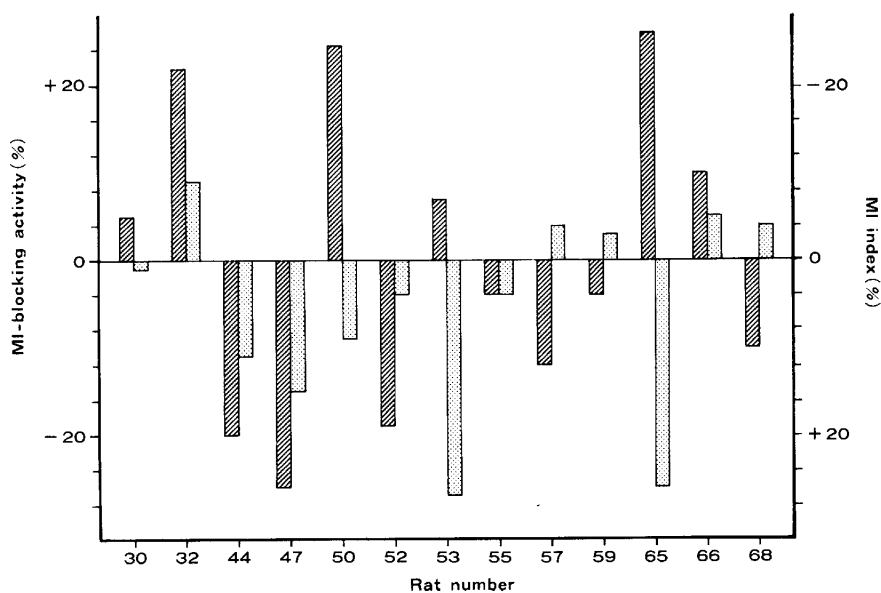b 'p' Value compared with Group 1 (c compared with Group 2)

Fig. 3. Blocking factor activity of macrophage migration inhibition in the sera of the Wistar recipients with long-surviving cardiac allografts. More than 20 % of MI indices were regarded as positive. Details in the text. ▨: MI blocking activity. ▦ : MI index.

before the allograft (p>0.05).
   Blocking factors in the sera from rats with long-surviving cardiac allografts.
   MI blocking activity.   MI blocking activity was examined in 13 rats among 15

TABLE 2. CYTOTOXIC INDEX AND SERUM BLOCKING ACTIVITY OF LMC IN THE WISTAR RECIPIENTS WITH LONG-SURVIVING CARDIAC ALLORRAFTS.

Rat no.	Tx	Cytotoxic index (%)	Serum blocking activity (%)
C_{20}	74	-52.2 ($p<0.01$)	-27.5 ($p<0.01$)
C_{27}	75	$+22.2$ ($0.05<p$)	$+38.4$ ($p<0.01$)
C_{30}	103	$+17.3$ ($0.05<p$)	$+7.7$ ($0.05<p$)
C_{32}	71	$+27.2$ ($p<0.01$)	$+61.6$ ($p<0.01$)
	135	$+33.7$ ($p<0.01$)	$+31.8$ ($p<0.01$)
C_{44}	121	$+15.4$ ($0.05<p$)	$+2.8$ ($0.05<p$)
C_{47}	120	$+2.7$ ($0.05<p$)	-23.5 ($p<0.01$)

LMC : Lymphocyte microcytotoxicity, Tx : Days after cardiac allograft

The rats with long-surviving cardiac allografts were examined. Significance test values are indicated in parentheses. Significant serum blocking activity was detected in rat No.27 and No. 32. In rat No. 32, the serum blocking activity was detected on the 71st and 135th days after the graft.

with cardiac allografts that survived more than 100 days. As shown in Fig. 3, MI blocking activity was detected in 6 rats (rat No. 30, 32, 50, 53, 65 and 66) out of the 13. The macrophage migration inhibitory factor (MIF) was positive in 2 rats (rat No. 53 and 65) among these 6 rats. On the other hand, the production of MIF tended to be high in the sera of 7 rats (rat No. 44, 47, 52, 55, 57 and 68), which had no blocking activity.

LC blocking activity. LC blocking activity was examined in 6 rats among the rats with cardiac allografts that survived a long time. As shown in Table 2, significant lymphocyte cytotoxicity was detected only twice in one rat although lymphocyte cytotoxicity was detected six times in the five other rats. Although serum blocking activity was detected 5 times in 4 of the 6 rats, significant activity was detected only three times in 2 of the rats. LC and MI blocking activities were simultaneously measured in rats No. 30, 32, 44 and 47. Both of the blocking activities were detected in rat No. 32, and the cytotoxic index (MI index) was increased by adding test serum of rat No. 47.

DISCUSSION

Immunological enhancement was originally defined in a tumor graft system (9). In the field of organ transplantation, immunological enhancement has been studied extensively since procedures for organ transplantation were established using the microvascular techniques of Lee (10) and Ono and Lindsey (2). Immunological enhancement is considered to increase the survival time of a graft due to the production of a specific antibody against the donor's histocompatibility antigen. Although resembling it, immunological enhancement is different from

immunological unresponsiveness, *i.e.*, classical tolerance, in that it concerns the recipient's immunoreactivity (11). Recently, however, immunological enhancement has tended to be regarded as partial tolerance, *i.e.*, transplantation tolerance rather than classical tolerance.

Although the induction and maintenance of enhancement are not well understood, the following points have been taken into consideration : disappearance of clones ; blockade, fixation and removal of receptors ; neutralization of antigens and feed back by antibodies ; production of serum blocking factors, and appearance of suppressor cells (12). Various other factors appear to be involved, such as enhancement of target cells and the method of induction itself. The following seem to be necessary for successful active enhancement in experimental animals : closely resembling major histocompatibility antigens, antigens which can produce the optimal amount of effective antibodies in recipients without stimulating transplantation immunity and suitable administration of the antigens (13).

As antigens for organ graft pretreatment, viable donor specific bone marrow cells, lymphnode cells or peripheral blood lymphocytes are used in semisoluble or soluble antigen form. Semisoluble antigen prepared from donor specific spleen cells was used in the present study because of the simplicity of its preparation and because the antigenicity is maintained during preparation of antigens. The antigen preparation which contains its essential components in an acellular, nontoxic, purified and stable form with high specific activity is desirable if immunological enhancement is to be clinically therapeutic (13).

As stated above, it is important to determine the optimal time of antigen pretreatment for the induction of active enhancement. Most previous reports, however, have discussed it based on the result of transplantation (3, 14), and few reports have determined the optimal time of antigen pretreatment prior to transplantation by studying the blocking activities. Stuart *et al.* (15) proposed 10 days before renal transplantation as the optimal time of pretreatment with antigen from results based on a passive hemagglutinin assay ; Rao *et al.* (16) 21 days before skin grafts from a cell mediated cytotoxicity test, and Guttmann (17) 5-14 days before renal grafts from leukocyte migration inhibition, lymphocytotoxic antibody, cell mediated cytotoxicity and mixed leukocyte culture tests. It is interesting that Guttmann (17) detected enhancing antibodies in the serum of a recipient having received antigen pretreatment 5 days before transplantation.

In this study the optimal time of antigen pretreatment was studied by monitering serum blocking factors using MIT after intravenous administration of the donor specific semisoluble antigens (2 mg/kg) to the recipient. Long survival of cardiac allografts was attained by pretreatment with the antigen at the optimal time, as confirmed by Guttmann *et al.* (18).

The optimal dose of semisoluble antigen in the present study was 2-4 mg/kg, which was less than that of other reports. Immunological enhancement was thought to be induced with less antigen since the antigen disparity between the

donor and recipient was small, namely, the rats were R antigen compatible and non-R antigen incompatible.

Although antigens were mostly administered only once in this study, there have been reports that administering them three times is better than only once (13, 18, 20). In this study a comparison between a single administration and three administrations showed no significant difference in the survival of cardiac allografts ($p < 0.05$).

Hellström et al. (21) detected the existance of factors in the sera of mice bearing MSV sarcomas, now called serum blocking factors (21, 22), which specifically inhibited the formation of lymphocyte colonies. Stuart et al. (22) found that the sera of rats bearing long-surviving kidney allografts specifically blocked the microcytotoxicity test in vitro. Recently there have been many reports of serum blocking factors or enhancing antibodies in the sera of animals and humans in which immunological enhancement was induced. However, there are differences among those reports in the frequency of detection and in the degree of activity of the blocking factors due to genetic disparity among the strains of animals used, immunocompetency of the host and different methods of detection. In this study, serum blocking factors were detected in 4 cases out of 6 cases (66.7 %) by the lymphocyte microcytotoxicity assay, and in 6 cases out of 13 cases (46.2 %) by the macrophage migration inhibition test.

It is said that there is a correlation between the presence of serum blocking factors and a well-adapted, functioning graft, but there is no correlation between their presence and long survival of a graft (23). It was reported that graft rejection occurred and blocking factors disappered in host sera when the state of tolerance was broken by administrating additional antigens (23).

Although it is not clearly known what serum blocking factors are, recent proposals are that they are soluble antigen-antibody complexes, soluble antigens, or antibodies themselves (24-27). The inhibition mechanism of the blocking factors has been divided into the following two components : target blocking in which the antigen-recognition site is blocked by antibodies and effector blocking in which antigens block effector cells.

Rocklin et al. (28, 29) detected the presence of MI blocking factors in the sera of normal multigravid women and pregnant women. According to their reports, MI blocking factors were 7S molecules as assayed by Sephadex G-200 gel chromatography and IgG antibodies stable at 56 ℃ for 30 min.

In order to make clinical use of immunological enhancement in organ transplantation, it will be necessary to have a much better understanding of the mechanisms which underlie the survival of transplanted organs. Therefore, further study on the blocking factors is also necessary in relation to anti-idiotypic antibodies (30) and suppressor cells (12).

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