STUDIES ON PARABIOTIC UNION

BY

Hiroshi IWASA, Yasuhiro OKAMOTO, Kanetoshi TAMURA,
Mitsuru TAKAHASHI, Ryoji HATANO
and Kohei NAGAKURA*

ABSTRACT

In the study on the homotransplantation of the skin, parabiotic union is frequently used. In the present work, parabiosis was carried out between the uniform strain of Wistar rats and uniform strain of Sprague-Dawley rats, and the cytotoxic antibodies were examined.

First, the skin of the Sprague-Dawley rat was transplanted to the Wistar rats and the antibodies were measured by Terasaki's improved microdroplet assay method for cytotoxin before the transplantation and also on the 3rd, 5th, 7th, and 10th day after the second transplantation.

The cytotoxic antibodies were not found before the transplantation but were observed with the course of time and also after the second transplantation.

The mechanism of the rejection of the transplant is still not clear, but the appearance of cytotoxic antibodies has been confirmed and is now regarded as important.

Therefore, an anti-rat-lymphocyte rabbit serum was prepared and an attempt was made to prolong the graft viability in parabiosis.

INTRODUCTION

From olden times, skin transplantation has been performed quite often as a kind of homotransplantation. There are various methods for homotransplantation. Parabiosis, a kind of a skin transplantation, is convenient for grasping clearly the rejection phenomenon. Therefore, the authors performed a parabiosis between a uniform strain of female Wistar rat and a uniform strain of female Sprague-Dawley rat, and examined the tissue homotransplantation.

Experimentally, the kidneys were the first organ on which homotransplantation was studied and since then many organs such as the liver, stomach, lungs, heart, spleen, intestine, etc., have been and are being studied. However, in the meantime, it was reported that the skin has a

* 岩佐博・岡本安弘・田村金寿・高橋満・畑野良侍・永倉幸平: 1st Department of Surgery (Chief: Prof. K. KAWASHIMA), School of Medicine, Tokyo Medical and Dental University (Tokyo Ika Shika Daigaku).

Received for publication, March 1, 1967.

strong antigenicity and easily produces a rejection phenomenon and, furthermore, parabiosis also shows a strong antigenicity. If by other new method the graft viability of the skin can be prolonged, then an effective method for inhibiting immunity can be hoped.

In the first place, as the present objective was the immunological tolerance when an antilymphocyte serum is used, it would be considered that the organ transplantation would be more likely to succeed if the duration of a parabiotic union can be prolonged.

Below is the report on the preliminary and main experiments on parabiosis.

Preliminary Experiment

(1) Cross hemodynamics

In conducting a parabiosis, the important point is whether the circulatory dynamics has been completed or not. There are various methods by which one can learn about the cross dynamics. In the present work, dyes and radioisotopes were used to measure the circulatory dynamics.

(a) Dye method

The distribution of Evans blue from one animal parabiont to the other was observed on the third day after the parabiosis. In measuring the duration of the presence of Evans blue after a single intravenous administration, it was found that when the dye was undiluted, it is present in the body for more than 3 days after its intravenous administration. Therefore, an undiluted dye is not appropriate, and 0.5 ml of a 20% dye solution is sufficient for this purpose. Further, it was learned that the dye is excreted from the body in 48 hours, and this method was mainly used (Table 1).

Table 1. Evans blue test

	A Group	B Group	C Group	D Group
	E-B 0.5	E-B 0.2 + Aqua 0.8	E-B 0.5 + Aqua 0.5	E-B 0.1 + Aque 0.4
30′	+	+	+	+
60′	+	+	+	+
24°	+	+	+	+
48°	+	+	+	−
72°	+	+	−	−

Unit: ml

(b) Radioisotope method

In studying the cross hemodynamics by using ^{59}F-labeled erythrocytes, it was found that there was a communication between the pair on the third day. The circulatory dynamics up to the tenth day, as shown in Fig. 1,

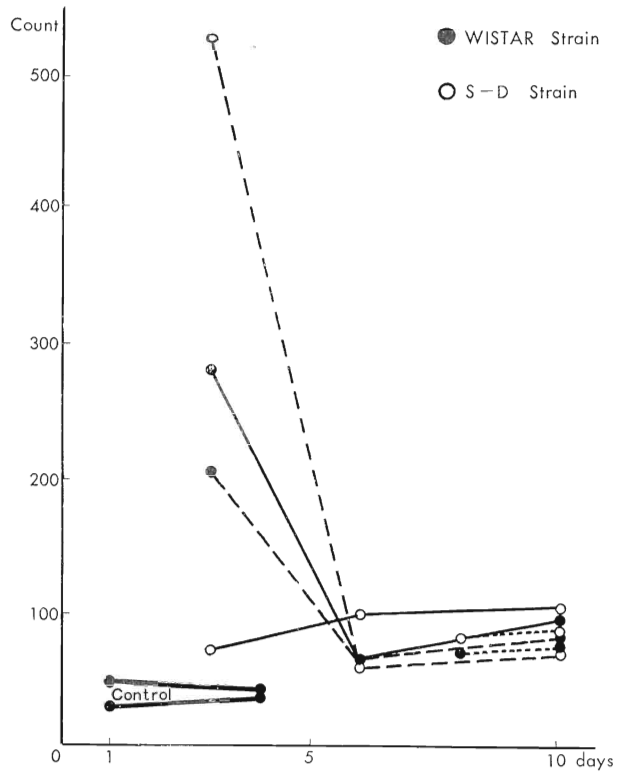


Fig. 1

indicated a comparatively high value on the third day and remained at the same values thereafter. As the half-life of ^{59}Fe is 45 days, the Evans blue method was better in observing the cross dynamics.

(2) Antilymphocyte serum

Experiments to destroy the leucocytes by immunological methods have been done from olden times. As early as in 1899, Metchnikoff immunized guinea pigs with lymph node cells or the cells of the spleen of the rabbit or rat and with the antiserum thus obtained tried to agglutinate or destroy the polymorphonuclear leucocytes of the donor. Later, many experiments have been carried out *in vivo* and *in vitro,* but it is only recently that this matter has become of interest in the sense that this can be used for the

inhibition of homotransplantation immunity. In 1964, Woodruff reported on the effect on the prolongation of the graft viability of the homotransplanted skin grafts, when the thoracic-duct fistule was made and combined with the injection of antilymphocyte sera. Following this, Hume of Virginia, Russell of Boston, and others reported on the results in rats and mice. In 1965, Fujimoto reported that he obtained sufficiently good results in rats, but that even the antilymphocyte serum had little effect on the transplantation immunity in dogs.

The present authors first prepared an antilymphocyte serum in order to examine whether it has any effect on the prolongation of graft viability in parabiosis, in which immunological tolerance is considered difficult to be produced.

(a) Method of preparing antilymphocyte serum

There are various methods reported on this. In the present work, the spleen and lymph nodes of a uniform strain of Wistar rats were passed through a screen made of stainless steel. The cell suspension thus obtained was washed well with dextran and the red cells were eliminated as much as possible. Freund's complete adjuvant was added to this cell suspension and the mixture was injected intradermally into the sole and back of the rabbit. Additional two injections of the above mixture of cell suspension and complete adjuvant were made subcutaneously at 10-day intervals. Then blood was drawn from the aural vein and the antibody titer was determined. When the antibody titer became high, the entire blood was drawn out and submitted to the determination.

The results of the latex agglutination test with this anti-rat-lymphocyte rabbit serum as shown in Table 2. The 1 : 16 positive serum was used.

Table 2. Agglutinin titer for latex-lymphocyte antigen

Serum dilution / Rabbit number	1	2	4	8	16	32	64	128	Cont
31	++	++	++	+	+	±	±	±	−
32	+	+	±	±	−	−	−	−	?
33	±	−	−	−	−	−	−	−	?

(b) Effect of anti-rat lymphocyte rabbit sera

In order to see if this serum is experimentally effective 0.5 ml of the antiserum was injected subcutaneously into the Wistar rats, which were divided into the multiple administration, single administration, and control groups, and then the lymphocyte count and hemogram were examined. The results are shown in Fig. 2. The lymphocytes decreased on the third

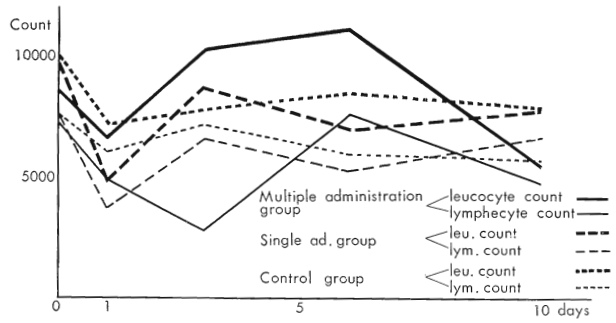


Fig. 2

day but, in the single administration group, the lymphocytes returned to the normal value on the fifth day. This fact indicates that a multiple administration is required to obtain any effect. Even in the multiple administration group, there was a tendency for temporary increase of lymphocytes after a decrease but this was considered to be due to the contamination of the antiserum by a mechanical error. The absolute number of the lymphocytes decreased from the tenth day by the administration of antilymphocyte serum.

(c) Mechanism of the action of antilymphocyte serum

The mechanism of this serum action is the destruction of the lymph cells. Medawar ascertained that the effect of the antilymphocyte serum in inhibiting immunity is marked. From this fact he stated that a strong blocking may occur in the proximal reaction arch where the antigen is transmitted to the reaction center. No doubt the lymphocyte count will return to the normal value before long after the administration is discontinued, and the length of administration is at the most about a week. If the transplanted tissue continues to be viable in spite of this, this phenomenon cannot be explained simply by the mechanism of lymphocyte destruction. If the administered heterogenous serum were to adhere to the surface of the lymphocytes of the host and to enclose the lymphocytes, the lymph cells of the host would not be able to react.

Moreover, by the action of the antilymphocyte serum, there is observed a strong inhibitory effect on the immunity even during the secondary transplantation. Therefore, in the case of kidney transplantation, when a small sign of rejection phenomenon appears, one can consider a method for overcoming the emergency with this antilymphocyte serum.

(3) Detection of cytotoxic antibodies

Cellular antibodies have drawn the attention as the new type of immunity. From the experiments by Medawar *et al.*, it has come to be

considered that usually only the cellular antibodies are related to the transplantation immunity and not the cytotoxic antibodies. However, with the advance in the serological method of examination, the cytotoxic antibodies which are humoral antibodies can also be detected. In regard to the relationship between the humoral and cellular antibodies, there is the question of whether a completely different immunological structure is present or antibodies produced by the same structure appear differently simply from the point of immunological phenomenon.

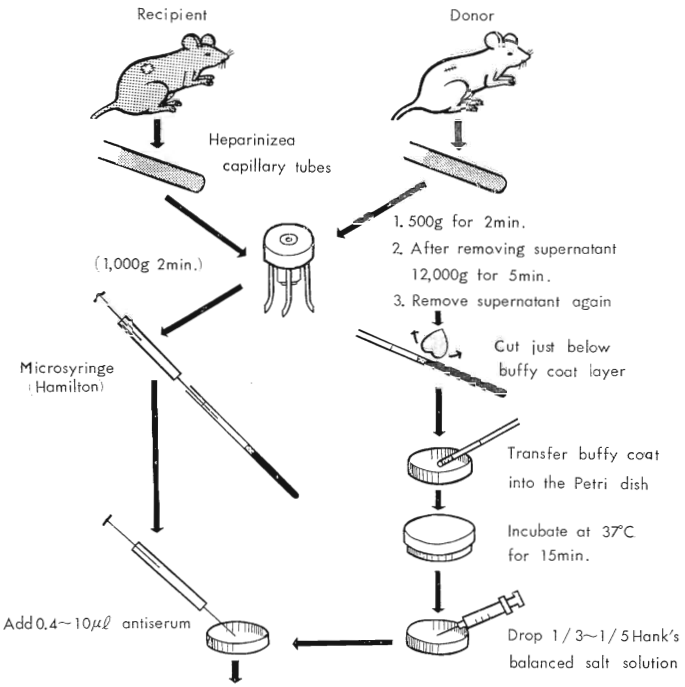
In the present work only the cytotoxic antibodies were examined and their relation to the antilymphocyte serum was studied.

(a) Terasaki's Modification

The cytotoxic antibodies were determined according to Terasaki's method. An outline of this method is shown in Fig. 3. A study was made as to see how the serum, antiserum, and complement of the donor act on the lymphocytes of the host. The inhibitory effect of the immunity was judged by the degree of the destruction of the host lymphocytes.

(b) Cytotoxic antibodies of rat skin homotransplantation

Using each 9 female Wistar and Sprague-Dawley rats, the skin, 2.0×2.0 cm in size, from the Sprague-Dawley rat was grafted to the side of the



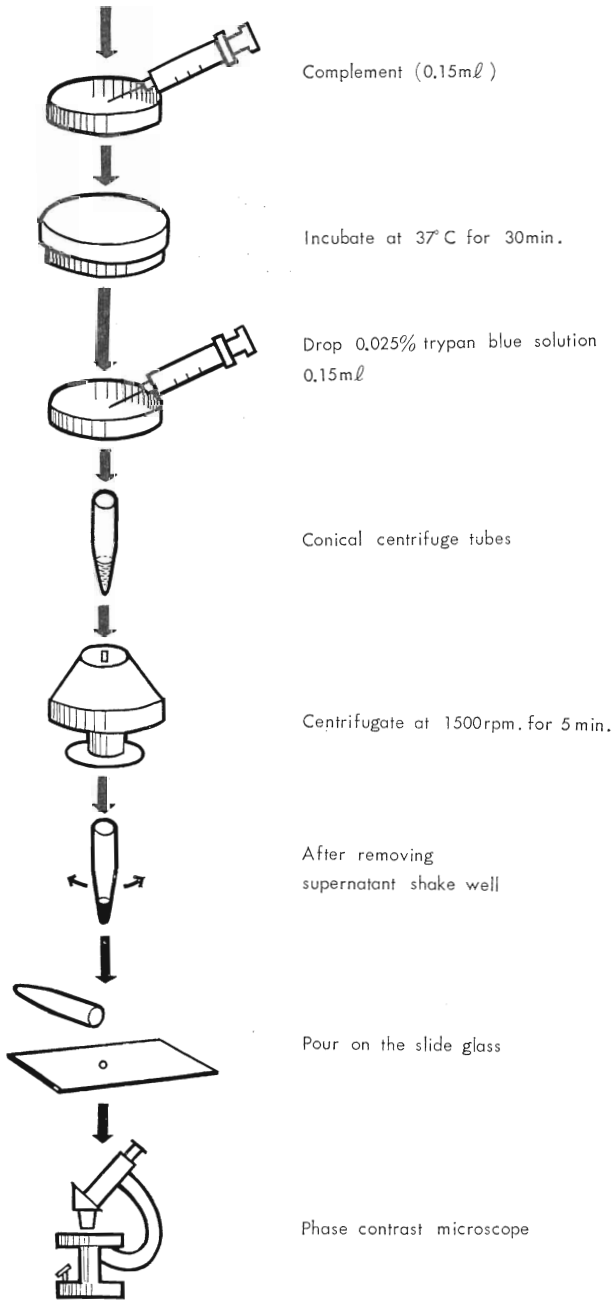


Fig. 3. Microdroplet assay of cytotoxin

abdomen of the Wistar rats. The course of rejection is shown in Fig. 4. In the first pair the graft was rejected on the tenth day and in the second pair on the eighth day. At this point, the cytotoxic antibodies were examined on the 7th, 10th, and 22nd day after the first graft on the Wistar rats, and on the 5th, 8th, and 20th day after the second graft.

In the present work the antibody titer is expressed by the number of nonviable lymphocytes per 1000 total lymphocytes by the microdroplet assay of cytotoxin (Tables 3 and 4, and Fig. 5).

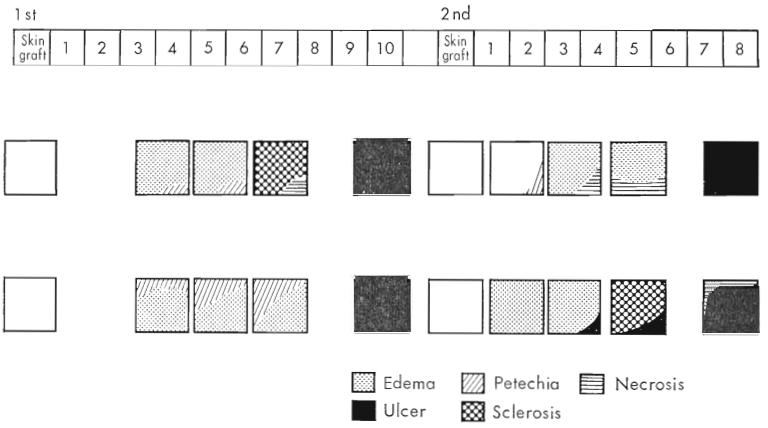


Fig. 4. Course of rejection phenomena

Table 3. Cytotoxic antibody (rat) (Terasaki's Method)

Ist skin homograft group

Serum dilution		1 : 1	1 : 5	1 :25
7 th day	Control	20.2	14.6	11.8
	15			
	18	18.5		
10 th day	Control	6.7	7.6	8.3
	15	28.3	11.2	20.2
	18	13.6	18.2	15.5
22 nd day	Control	2.9	2.2	5.7
	15	24.3	7.6	17.2

Number of nonviable lymphocytes for 200 lymphocytes

Table 4. Cytotoxic antibody (rat) (Terasaki's Method)
IInd skin homograft group

Serum dilution		1 : 1	1 : 5	1 : 25
5th day	Control	20.2	14.6	11.8
	11	17.4	14.6	33.1
	12	36.5	31.9	28.6
	13	54.1	21.1	30.8
	14	12.5		
8 th day	Control	6.7	7.6	8.3
	11		15.0	
	12	36.6	8.2	
	13	15.7	10.4	
	14	28.3	15.2	14.3
20th day	Control	2.9	2.2	5.7
	11	16.7	4.5	21.6
	12	13.8	5.8	21.5
	13	28.2	24.3	10.0
	14	11.0	15.5	3.6

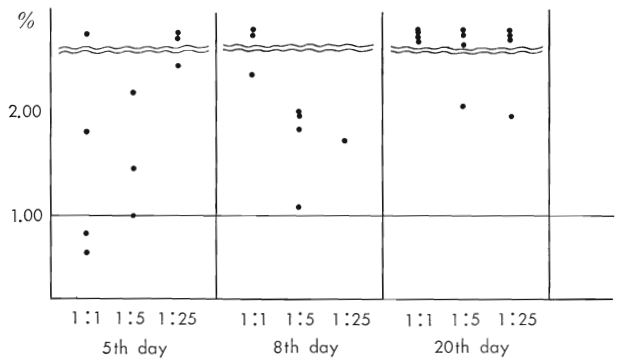


Fig. 5

As the results in Table 4 show, the value of the cytotoxic antibodies increased after the graft as compared to the control, but the value in the second pair was not higher than that of the first pair. Also, it was impossible

to conclude whether these values are proportional directly to the antibody titer of the graft.

(c) Cytotoxic antibodies of skin homotransplantation in the rabbit

The same experiment was conducted with non-inbred strain rabbits, as on the rats. When the skin of the size of 2.5×2.5 cm taken from the back of a non-inbred strain rabbit was grafted to the back of another non-inbred rabbit, a rejection was observed. The blood was drawn and the cytotoxic antibodies were measured. As shown in Table 5, the antibody titer was

Table 5. Cytotoxic antibody (rabbit) (Terasaki's Method)

Serum dilution		1 : 1	1 : 5	1 : 25
Ist skin homograft group	Control	12	9	9
	I	46	72	43
	II	14	20	24
IInd skin homograft group	Control	15	15	20
	I	55	45	04
	II	40	20	50

Number of nonviable lymphocytes for 1000 lymphocytes

definitely hihger after the graft, but the titer of the second pair was not higher than that of the first pair.

Main Experiment

(a) Parabiosis

In parabiosis, the Wistar and Sprague-Dawley rats were joined together without any treatment. As shown by the broken lines in Fig. 6, there was a tendency for the Sprague-Dawley rats to die earlier. When one or the other of the pair died and the animals were separated early, some animals did not die, but after a while the remaining animal died due to parabiotic intoxication.

(b) Action of anti-rat-lymphocyte rabbit serum

The Wistar rats were given injection of 0.15 ml of the antiserum, repeatedly in the first group, and as a single injection to the second group, while no injection was given to the third group. Parabiosis was made with nontreated Sprague-Dawley rats.

The survival curve is shown in Fig. 6. The single administration and control groups showed the same tendency while the group administered

repeatedly with the antilymphocyte serum survived somewhat longer than the other two groups but the number of long survival cases was not as great as that anticipated. In all these cases, the cytotoxic antibodies were not detected by Terasaki's method.

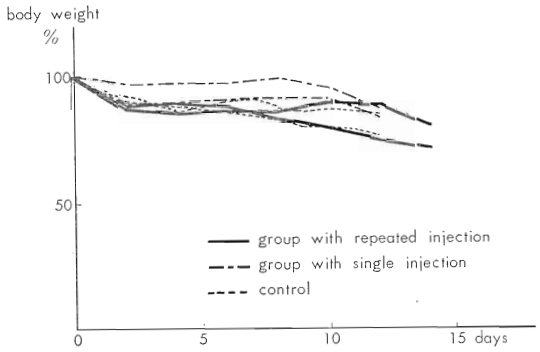


Fig. 7

Conclusion

Schwartz and Dameshek made a historic report in 1959 on the effect of 6-mercaptopurine and referred to the immunological tolerance produced by this chemical, stating that this tolerance was specific.

However the effective immunity-inhibiting agents known to this date are more or less toxic. Actually it is desirable to have a chemical with a considerable difference between the effective and toxic doses and examinations were made the antilymphocyte serum.

Fujimoto conducted a skin homotransplantation on the mice on the second to third day after the administration of an antiserum and the administration of the serum was continued for a total of 9 injections. He reported that the rejection of the grafted skin occurred on the 9.4th day in the nontreated control group, but in the group administered with the antilymphocyte serum the rejection did not occur on the average till the 15th or 16th day, stressing the effectiveness of the serum.

The anti-rat-lymphocyte rabbit serum was also prepared and used in the present work and, although anticipated results have not been observed as yet, a new field of research is expected to be developed in the near future.

Acknowledgements

The authors are indebted to Professor K. Kawashima for his continuous guidance during the course of this study. They also wish to extend their

appreciation to Dr. H. Hasekura, Department of Legal Medicine of this School for his useful advice and encouragement.

References

1) Fujimoto, Y.: "Organ transplantation." (in Japanese) Chugaiigaku, Co., Tokyo, 1965.
2) Terasaki, P. I., and McClelland, J. D.: Nature, **204**, 998–1000, 1964.
3) Kapitchnikov, M. M. *et al.*: Ann. N. Y. Acad. Sci., **99**, 497–503, 1962.
4) Hardin, C. H.: Surg. Forum, **12**, 103–105, 1961.
5) Streilein, J. W., *et al.*: J. Amer. Med. Ass. **195**, 351–355, 1966.
6) Billingham, R. E. *et al.*: Nature, **172**, 603–606, 1953.
7) Martinez, C., *et al.*: Proc. Soc. Exptl. Biol. Med. **102**, 413–417, 1957.
8) Najarian, V. S., *et al*: Surg. Forum, **15**, 127–128, 1964.
9) Mila, N. S.: Lancet, **7422**, 1130, 1965.
10) Dickson, J. A.: Brit. Med. J., **1**, 817–822, 1966.
11) Clarke, C. A., *et al*: Brit. Med. J., **1**, 509–513, 1966.
12) Najarian, J. S., *et al*: J. Exptl. Med., **121**, 521–532, 1965.
13) Yunis, E. J., *et al*: J. Exptl. Med. **121**, 607–632, 1965.
14) Pichlmaier, H., *et al*: Klin. Wschr., **44**, 57–67, 1965.
15) Russell, P. S., and Monaco, A. P.: "The Biology of Tissue Transplantation", Little, Brown and Company, Boston, 1964.
16) Moore, F. D.: "Give and Take", W. B. Saunders Company, Philadelphia and London, 1964.