195

THE COMPARATIVE IN VITRO AND IN VIVO ACTIVITY OF ANTILYMPHOCYTE SERUM RAISED BY IMMUNIZATION WITH THYMIC, SPLENIC, AND LYMPH NODE LYMPHOCYTES

KEIJI ONO, M.D.
PETER BELL, M.D.
NOBORU KASHIWAGI, M.D.
THOMAS E. STARZL, M.D.
Denver, Colo.

From the Department of Surgery, University of Colorado School of Medicine, and the Veterans Administration Hospital

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The comparative in vitro and in vivo activity of antilymphocyte serum raised by immunization with thymic, splenic, and lymph node lymphocytes

KEIJI ONO, M.D.
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DENVER, COLO.
From the Department of Surgery, University of Colorado School of Medicine, and the Veterans Administration Hospital

Although antilymphocyte serum (ALS) and antilymphocyte globulin (ALG) have been used extensively in clinical organ transplantation, the methods used to prepare and assay these agents have not yet been well standardized. For example, it has frequently been speculated that the immunosuppressive qualities of an ALS might vary significantly depending upon the type of lymphoid tissue used to immunize the heterologous serum donor.[1, 2, 5, 9, 11]

In the present study this hypothesis has been examined by comparing the ability of rabbit antilymphocyte sera raised against splenic, thymic, and lymph node lymphocytes to prevent the rejection in rats of heterotopically transplanted cardiac homografts. The antisera proved to have essentially identical immunosuppressive potencies. However, there were significant differences in the side effect of thrombocytopenia which was most consistently and markedly produced by the antispleen serum.

METHODS

Raising of ALS. The lymphoid tissue was taken from inbred Fischer (Fi)* rats (AgB 1/1) and was divided into tiny pieces by microdissection under normal saline. The cells were removed by passing them through a stainless steel mesh (60 denier) and a glass wool filter. The composition of the eventual antigen suspensions is given in Table I. It will be noted that the material derived from the spleen contained the greatest number of thrombocytes.

The cell suspensions were intravenously injected once a week for 4 weeks into 18 white New Zealand rabbits which were separated into three groups of six according to the lymphoid antigen being used. Each rabbit received the same number of lymphocytes in the course of the immunization, totaling 1.3×10^9 cells. Serial leukoagglutinin titers were obtained during this interval, and at its end the rabbits were bled by cardiac puncture. The sera from the three groups of six rabbits were pooled and decomplemented by heating at 56° C. for 30 minutes.

Absorption. The decomplemented antisera were first absorbed with one-third volume triply washed and packed rat erythrocytes. The three red cell absorbed pools were then each divided; half of the serum was used for definitive testing without further alteration. The other half in each subgroup was absorbed 3 times with platelets utilizing 5×10^8, 2.4×10^8, and 12×10^8 platelets per milliliter of serum. The mixtures

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Table I. *Cellular composition of antigen suspensions used for immunization*

Rat cell suspensions	Differential counts (%)			Thrombocytes/10^6 WBC
	Small lymph	Large lymph	Poly	
Spleen	90-94	3-5	3-5	12,500
Thymus	93-97	3-7		7,250
Lymph node (submaxillary and mesenteric)	90-92	8-10		2,000

of sera and platelets were left standing at 4° C. overnight in the first absorption but were gently agitated overnight during the second and third absorptions.

By this stage, six test sera were ready. In addition, normal rabbit serum (NRS) was collected for control studies; it was heated, and stored in the same manner as described but it was not absorbed with rat erythrocytes and thrombocytes.

In vitro assay. Lymphoagglutinin and thromboagglutinin titers were determined by placing 0.025 ml. of the serially diluted sera with the same volume of a lymph node lymphocyte (2×10^7 per milliliter) or thrombocyte (4×10^8 per milliliter) suspension. The mixture was made in a microtiter plate. Lymphoagglutination was read after 2.5 hours' incubation at 37° C. and thromboagglutination after 6 hours. Thymoagglutination titers were determined by the double dilution tube method. Thymocyte suspensions (1×10^7 per milliliter) of 0.1 ml.) were mixed with 0.1 ml. undiluted or diluted ALS in each tube and incubated at 37° C. for 60 minutes. All agglutination tests were considered as positive only if more than half of the cells were clumped. Lymphocytotoxicity was measured by the dye exclusion technique described elsewhere.[6]

In vivo assay. The effects of each of the seven different rabbit sera were measured in five young outbred Sprague-Dawley rats.* The rats were given intraperitoneal injections of 0.5 ml. on Days 0 and 2 and 1.0 ml. on Day 4. Rats were bled from the tail on Days 0, 1, 3, 5, 8, and 11, and red cell, total and differential white cell, and thrombocyte counts were determined. In addition the animals were weighed after each bleed.

*Sprague-Dawley, Inc., Madison, Wis. 53711.

The donors for the cardiac homografts were inbred adult Wistar-Furth (WF) rats* (AgB2/2). The aorta and pulmonary artery of the transplanted hearts were attached to the abdominal aorta and inferior vena cava, respectively, of the adult recipients by a previously described technique.[7] The adult recipients were all of the Fischer (AgB 1/1) strain. They were given 1.0 ml. intraperitoneal serum injections on Days 0, 2, and 5. Graft function was assessed by daily palpation and by biweekly electrocardiograms. Graft rejection was taken as complete absence of electrical activity of the transplant.

RESULTS

The serum titers. The antilymphocyte activity of all six test sera was exactly the same (Table II). However, there were significant differences in the thromboagglutinin titers. The highest antiplatelet activity was in the ALS that had been raised with splenic antigen (Table II). The antithrombocyte titers were reduced by the triple platelet absorption without affecting the antilymphocyte activity (Table II).

In vivo effects. There was no difference among the six test antisera in their effects on the white counts. The total lymphocyte counts were depressed immediately after the first intraperitoneal injection and did not begin to recover until many days after the last dose was given (Fig. 1). There was also a significant fall of the total white count of which most was accounted for by the loss of lymphocytes (Fig. 1). However, there was also a slight depression of the granulocyte counts.

The three unabsorbed test sera had variable effects on the circulating platelets. The

*Microbiological Associates, Inc., Bethesda, Md. 20014.

Table II. *In vitro activity of rabbit antirat lymphoid cell serum*

Sera	*Lymphoagglutinin*	*Thymoagglutinin*	*Lymphocytotoxicin*	*Thromboagglutinin*
RARSS*	1:1,000	1:1,000	1:512	1:64
RARTS†	1:1,000	1:1,000	1:512	1:32
RARLNS‡	1:1,000	1:1,000	1:512	1:16
RARSS-Abs.§	1:1,000	1:1,000	1:512	1:4
RARTS-Abs.	1:1,000	1:1,000	1:512	1:2
RARLNS-Abs.	1:1,000	1:1,000	1:512	1:2

*RARSS—rabbit antirat spleen cell serum.

†RARTS—rabbit antirat thymus cell serum.

‡RARLNS—rabbit antirat lymph node cell serum.

§Abs.—absorbed with rat thrombocytes.

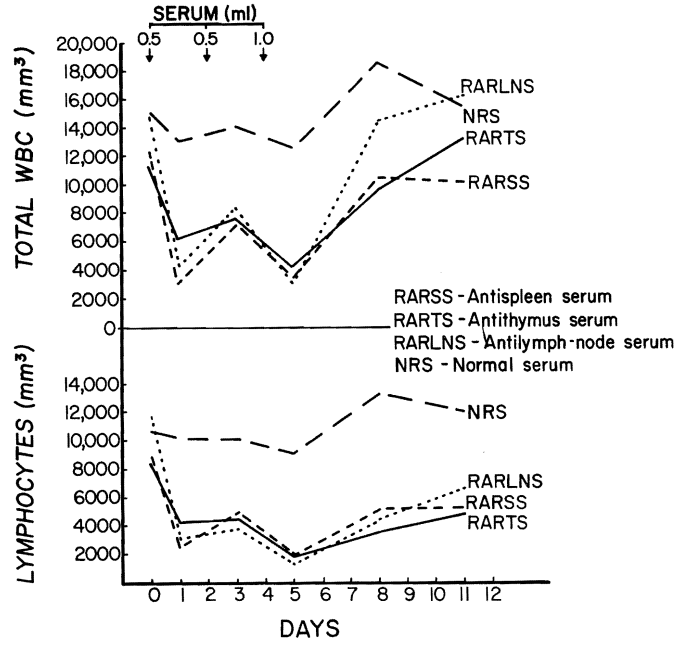

Fig. 1. The effect of normal, antithymic, antisplenic, and antilymph node sera on the total peripheral white cell counts and the lymphocyte counts of Sprague-Dawley rats. The antisera were raised in rabbits.

ALS raised with spleen caused the most profound thrombocytopenia. The antithymocyte serum caused almost as severe changes. The antilymph node serum had the least effect (Fig. 2). After absorption with platelets, none of the ALS's produced thrombocytopenia (Fig. 2).

An interesting finding was the discrepancy of the body weight gain of rats treated with the various sera. The animals treated with NRS gained 53 percent body weight for 11 days, while other groups had 21 to 36 percent gain. The rats given ALS raised with splenic antigen had diarrhea for several days and their weight gain was only 21 percent.

Cardiac transplantation. In the rats treated with normal rabbit serum, the heterotopic cardiac grafts remained viable for a mean of 12 days. In contrast, the transplanted hearts in animals treated with ALS had a mean survival of more than a month ($p < 0.001$). There was no significant dif-

Table III. *Survival of rat heart allografts treated with rabbit antirat lymphoid cell sera*

Sera	*No. of grafts*	*Range (days)*	*Mean S.D. (S.E.) (days)*	*p Value*
Spleen	10	28-41 28, 30, 30, 33, 33, 34, 35, 37, 38, 41	33.9 ± 3.9 (1.3)	$p<0.001$
Thymus	10	20-40 20, 23, 24, 27, 33, 34, 35, 38, 39, 40	31.3 ± 6.7 (2.4)	$p<0.001$
Lymph node	10	27-46 27, 30, 31, 31, 34, 39, 41, 41, 44, 46	36.4 ± 6.3 (2.2)	$p<0.001$
Normal rabbit serum	10	9-15 9, 10, 10, 11, 12, 12, 13, 13, 15, 15	12.0 ± 1.9 (0.7)	

Recipient rats were given 1 ml. of each serum on Days 0, 2, and 5 intraperitoneally.

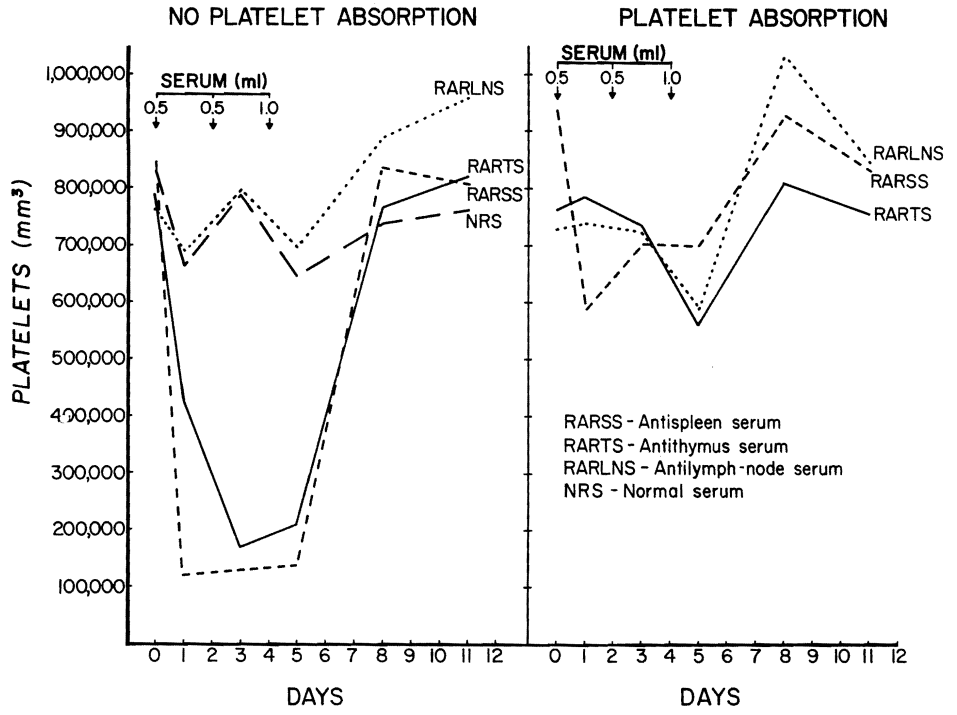

Fig. 2. A comparison of the platelet depressing effect of different kinds of rabbit antirat ALS (left). On the right are repeat studies after the different kinds of ALS had been absorbed with rat platelets. The test animals were Sprague-Dawley rats.

ference in the protection of the transplants by antisera raised with thymic, splenic, or lymph node antigen (Table III).

DISCUSSION

There were three features of the protocol in this study which made it possible to compare the qualities of different kinds of ALS with a high degree of accuracy. The first was that the use of a controlled inbred rat strain (Fi) as the sole source of lymphoid tissue for the rabbits assured genetic homogeneity of the immunizing antigen. Second, the immunologic barrier across which the transplantations were carried out was a well-defined one (AgB 2/2 → AgB 1/1)

which is comparable to the H-2 system in mice. Consequently, the data in both the control and treated recipients were highly reproducible. Finally, the difficulty often encountered with skin grafts in defining the exact time of rejection was eliminated. There was no equivocation about when the heterotopic hearts ceased to beat and to emit organized electrical activity.

The results obtained under these conditions provided no evidence whatever that a heterologous ALS with inherently superior immunosuppressive properties could be raised with any particular kind of lymphoid tissue. On the contrary, the lymphopenic effects of the antithymic, antisplenic, and antilymph node sera were almost identical. Moreover, the ability of the agents to forestall rejection of auxiliary heart grafts did not differ significantly.

Nevertheless, the experiments emphasized that the need for troublesome absorption procedures was influenced by the tissue used for immunization of the rabbits. When spleens were used, undesirable antiplatelet antibodies were evoked in the highest titer. This effect was somewhat less with thymuses and it was barely discernible with lymph nodes. As Kashiwagi and associates[3, 10] have also reported, the thromboagglutinins could be removed from ALS by absorption procedures without demonstrably affecting the antiwhite cell titers. However, the number of platelets required for this purpose was so large that a considerable expense would be involved for the commercial production of bulk quantities of the antisera.

The thrombocytopenia produced in rats when the unabsorbed antispleen and antithymus antisera were given intraperitoneally was not serious enough to adversely affect the results after cardiac transplantation. The reason apparently was that the therapeutic course was limited to only three injections in 5 days. During this interval or afterward obvious bleeding diatheses were not observed.

In clinical practice, the implications of platelet depression have been less trivial in patients receiving ALG prepared with splenic antigen. In a trial in which it was attempted to give the immune horse globulin daily in large quantities for several weeks after renal transplantation in an effort to produce early tolerance, the limitations of permissible dosage were imposed by the extent of the induced thrombocytopenia.[10] When this complication occurred, it was necessary to reduce both the frequency and volume of the planned injections. The consequent need to give the ALG as intermittent therapy rather than continuously was particularly disquieting since the "pulse" technique is known to increase the rapidity of sensitization of the treated subject to the foreign protein and to therefore shorten the period of the immunosuppressive efficiency.[4, 6]

It may be that some degree of platelet depression must be accepted as the inherent penalty for high dose ALG treatment since studies from both the Munich laboratories of Pichlmayr and associates[8] and our own[10] have shown the cross-reactivity of platelets and lymphocytes to ALS. However, the data in the present study suggest that the predominant thrombocytopenic effect of an ALS is due to thromboagglutinins caused by platelets which were accidentally included with the immunizing lymphocytes.

It follows that there are two specific measures which can be taken to decrease the thrombocytopenic effect of the ALS raised for human use. The most obvious is to take special pains to remove the platelets as completely as possible from the cell suspensions administered to the heterologous serum donor. Elimination of these platelets can be accomplished by filtration, differential centrifugation, and other mechanical means. In addition, it is of the utmost importance to check the ALS for antiplatelet activity before the globulin extraction stage is begun. Even if a seemingly insignificant activity is found, absorption should be carried out with platelets since thrombocytopenia has been caused in patients with ALG that had thromboagglutinin titers as low as 1:4. In the present study, immediate platelet de-

pressions occurred with antisera that had thromboagglutinin titers of 1:32 or less.

SUMMARY

Rabbit ALS was raised against the splenic, thymic, and lymph node lymphocytes of inbred Fischer rats. The different antisera had the same ability to induce lymphopenia or to protect auxiliary cardiac homografts from rejection after transplantation from Wistar-Furth donors to Fischer recipients. There was a difference in the toxicity of the agents in that the antispleen and antithymus sera caused thrombocytopenia. The severity of this complication seemed related to the degree of platelet contamination of the rat cell suspensions originally given to the rabbits. The thrombocytopenia can be at least partially avoided by cleaning up the immunizing antigen as well as by platelet absorption of the resulting ALS.

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