FEDERATION PROCEEDINGS
Vol. 29, No. 1, January-February, 1970
Printed in U.S.A.

Selected topics on antilymphocyte serum[1]

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AT A SIMILAR MEETING in June of 1966, also held at Brook Lodge, we presented data from more than 200 canine experiments which were designed (8, 26, 27) to determine: *1*) what magnitude of immunosuppression could be expected in dogs from treatment with small volumes of antilymphocyte sera (ALS) possessing high antiwhite blood cell titers, *2*) what were the toxic effects of this kind of therapy, and *3*) what were acceptable methods by which the globulin derivative (ALG) of ALS could be used clinically. The way in which the results of the animal investigations were translated into a clinical treatment protocol was also discussed at the same conference based upon the first experience with human recipients of renal homografts who were treated with adjuvant horse immune globulin.

Including these early patients, we have now given intramuscular ALG, in combination with azathioprine and prednisone, to about 140 recipients of kidneys, livers, or hearts. The injections have not been directly responsible for any deaths. The benefits as well as the side effects of ALG have been analyzed from our case material on a number of occasions (11, 25, 27, 28) and will not be repeated here. Suffice it to say that after the addition of horse ALG to the therapeutic regimen in our institutions, the 1-year survival after intrafamilial renal transplantation rose to more than 90 % and that after cadaveric renal transplantation to 83 %. In some of the liver recipients, of whom four have now lived for more than a year after operation, azathioprine was very greatly reduced and reliance was placed mainly on ALG and prednisone.

Since ALG has been shown to be a potent immunosuppressive agent in every species including man in which it has been tested appropriately, there seems little reason to debate this issue in connection with organ transplantation in humans. However, a more legitimate inquiry has been raised by Hume et al. (7), namely, whether ALG is really needed in addition to azathioprine and prednisone for kidney transplantation, at least in their center. We will not dwell on this question since it has little to do with the points we wish to discuss here which are very simple and only three in number.

[1] This study was supported by Public Health Service Grants AM-06344, AM-07772, FR-00051, AI-04152, FR-00069, AM-12148, and AI-AM-08898.

IMMUNOSUPPRESSION OF AN ALS IN RELATION TO THE IMMUNIZING LYMPHOID ANTIGEN

It has often been speculated that an ALS with superior immunosuppressive qualities might be produced with special lymphoid antigen of some kind or other (2, 9, 16, 21, 30); the thymus gland and thoracic duct lymphocytes seem to have been sentimental favorites.

Ono and his associates have been the only ones to formally examine this hypothesis (17). They raised ALS in New Zealand rabbits with identical doses and injection schedules of lymphocytes obtained from the spleens, thymus glands, and lymph nodes of inbred Fischer rats. The three different antisera all had exactly the same lymphoagglutinin (1:1,000), and lymphocytotoxicity titers (1:512). The thromboagglutinin titers were the highest in the antispleen (1:64) and antithymus sera (1:32) and lowest in the antilymph node serum (1:16). This was explained by the fact that the injections of the splenic and thymic lymphocytes had been more heavily contaminated with rat thrombocytes than was the case with the immunizing lymph node cell preparations.

When the different kinds of rabbit antisera were injected intraperitoneally in rats, almost precisely the same degree of lymphopenia was produced. However, there was a difference in the side effect of thrombocytopenia. This was minor with the sera raised with lymph node lymphocytes, but it was moderately severe with both the antispleen and antithymus sera. The thrombocytopenic effect could be readily removed from the latter ALS preparations by absorption with rat platelets. The lymphopenic and immunosuppressive effects were not thereby reduced.

The ability of the three different kinds of ALS to prevent rejection was assessed in a model which permitted very precise delineation of results. The hearts of adult Wistar-Furth rats (AgB 2/2) were transplanted to the abdomens of Fischer strain rats (AgB 1/1), anastomozing the graft aorta and pulmonary artery to the recipient aorta and inferior vena cava, respectively. There was no equivocation about the diagnosis of end-stage rejection since this was defined as the time when a palpable heart beat stopped and when organized electrical activity ceased as measured by the EKG. Control animals were given 1 ml normal rabbit serum intraperitoneally on

TABLE 1. *Survival of rat heart allografts treated with rabbit antirat lymphoid cell sera*

Sera	Number of Grafts	Range (days)	Mean SD (SE) (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.

days 0, 2, and *5*. The same therapeutic schedule was followed with the three antisera.

Across this strong histocompatibility barrier, the mean survival of the heart grafts was 12 days in the control animals. This was increased to more than a month by three injections of each of the antilymphoid sera (Table 1). There was no significant difference in the outcome using the various immune sera.

The conclusions from this study were straightforward. First, there was no special advantage of using any particular kind of lymphoid tissue for immunization, at least in terms of the ultimate immunosuppressive effect. Second, the undesirable thrombocytopenia produced by some of the antisera was related directly to the platelet contamination in the cell suspensions used for the immunization. Finally, most of the antiplatelet activity could be removed by absorption procedures without perceptibly reducing the ability of the sera to mitigate rejection.

Since absorption with platelets is a time consuming and expensive step in the commercial preparation of ALS, a preferable solution is to take special pains to remove the platelets as completely as possible from the cell suspensions administered to the heterologous serum donor. This has been accomplished in our laboratory with splenic antigen by filtration, by differential centrifugation, and by other mechanical means. 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 Pichlmayr's laboratories (20) and our own (24) have shown some cross reactivity of platelets and lymphocytes to ALS. However, the data in Ono's study suggest that the predominant thrombocytopenic effect of an ALS is explicable by thromboagglutinins caused by platelets which were accidentally included with the immunizing lymphocytes.

LOCALIZATION OF ALS ANTIBODIES

In the immunized rabbit, there has been little dispute that the antiwhite cell antibodies and the immunosuppressive effect reside exclusively or nearly so in the gamma-G globulin (10, 12–15, 29); the same apparently is true in the goat (12). However, horse ALS has been reported by several authors to have a greater spread of the activity within the 7S gamma-globulin during the first few months of lymphoid tissue administration (3, 8, 10, 19, 20, 24). Moreover, at a later time, after 3–11 months of immunization, Pichlmayr described a shift of the antiwhite cell antibodies to the IgM fraction (19). Because of the loss of immunosuppressive potency of highly refined equine IgG that has been reported (4, 24), Kashiwagi (12) undertook a reevaluation of the antibody localization in horse ALS using a combination of analytic techniques that included column chromatography, electrophoresis, immunoelectrophoresis, determination of protein concentration, and measurement of antibody titers.

The three horses studied by Kashiwagi had been immunized weekly with subcutaneous canine or human spleen cells for 6–9 weeks; they were considered to be "early" serum donors. The results were the same with the sera of all three animals. There were seven protein peaks (fractions) identifiable by chromatography. The lymphocytotoxic activity was very heavily, if not exclusively, concentrated in the IgG. This was particularly evident in the pooled fractions I and II (Fig. 1) in which only IgG was represented. The lymphocytotoxins in the fractions III and IV were probably also contained in the IgG which contributed to these heterogenous collections.

In contrast, more than 80% of the leukoagglutinins were in the fractions III and IV (T-equine globulin) which consisted predominantly of IgA (also known as "fast" gamma-G, IgG (T)). The hemagglutinins and thromboagglutinins were mostly in the same location, although these antibodies were also detectable in the IgG (Fig. 1).

The differential ammonium sulfate precipitation of Allen (1) was used to obtain bulk quantities of two horse antidog lymphocyte globulins (ALG). One, called fraction G, had a high concentration of IgG. The other which was considerably more heterogenous was termed fraction T; its principal immunoglobulin was IgA. Before testing fraction G, it was diluted to give the same gamma-globulin concentration as in the original ALS. A similar adjustment was made for the T-equine content of fraction T.

The protein concentrations and the antibody titers of the adjusted fractions are shown in Table 2, as well as the results obtained by subcutaneously giving 0.5 ml/kg per day of these substances to mongrel canine recipients of nonrelated kidney transplants. Treatment was started the day before operation. The survival was prolonged under therapy with the ALS and the fractions G and T removed from it. There seemed to be no difference using ALS and fraction T ($P > 0.1$) whereas the animals injected with fraction G lived for somewhat shorter periods ($P < 0.01$).

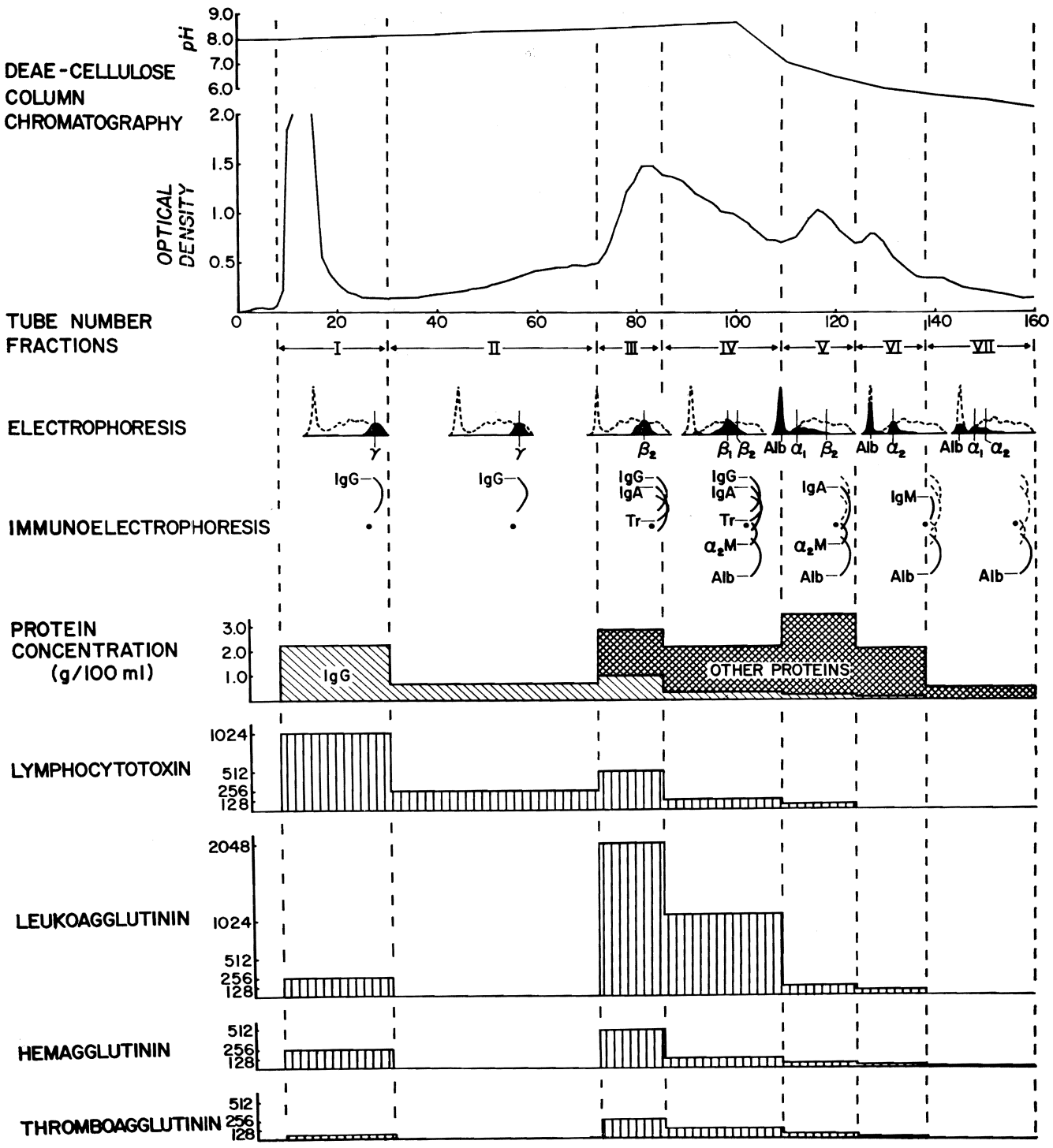

FIG. 1. Analysis of the location of antibodies in the serum of a horse that had been immunized for 6 weeks with weekly subcutaneous injections of human splenic cells. Adjuvant was not used in this or any of the other heterologous serum donors. The multiple analytic techniques shown were used to determine the varieties, concentrations, and activities of the proteins in the different fractions. Alb, Albumin; α_1, alpha$_1$-globulin; α_2, alpha$_2$-globulin; α_2M, alpha$_2$-macroglobulin; β_1, beta$_1$-globulin; β_2, beta$_2$-globulin or T-equine; γ, gamma-globulin; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; Tr, transferrin.

These studies have not weakened the proposition that most of the active portion of equine ALS is 7S gamma-globulin in the first months after the beginning of immunization. However, they do provide a possible explanation for the loss of potency seen with highly refined horse IgG since this portion ("slow" gamma-G) represents only part of the total activity. The other portion consists of the "fast" gamma-G (IgA) so called because of its slightly greater electrophoretic mobility. The IgA, which is buried in the T-equine globulin, is

TABLE 2. *Results with canine renal transplantation using whole horse ALS or globulin fractions*

	Protein Concentration, g/100 ml				Antibodies				No. Dogs	Survival			*P* Value	
	Gamma	T-equine	Beta1, alpha, and albumin	Total	Leukoagglutinin	Lymphocytotoxin	Hemagglutinin	Thrombo-agglutinin		Mean	Range	SE(I)	To Control	To ALS
Absorbed ALS	1.7	1.6	3.8	7.1	1:2048	1:512	1:128	1:32	6	20.6	10–50	7.5	<0.01	
Fraction G	1.9	0.7	0.4	3.0	1:512	1:512	1:128	1:16	10	16.3	9–34	3.3	<0.01	<0.01
Fraction T	0.7	1.6	2.6	4.9	1:2048	1:256	1:128	1:32	10	19.4	11–35	3.3	<0.01	>0.1
No treatment									11	9.9	5–21	3.5		

By concentration or dilution, it was attempted to give the fraction G injections the same gamma-globulin content as that in the ALS doses. A similar adjustment was made for the T-equine content of the fraction T. Note the high degree of contamination of the fraction T with extraneous protein.

TABLE 3. *Cases of new malignancy developing in recipients of renal homografts**

	Epithelial	Mesenchymal	Total
Total	6	9	15
Azathioprine	6	9	15
Prednisone	6	9	15
ALG	0	3†	3

* Eight cases have been reported in the literature (5, 6, 8, 18, 22, 23, 31). Details of the other cases were obtained by personal communication. † Woodruff's patient received ALG but only after the thoracic reticulum cell sarcoma was already visible on the chest X-ray.

discarded in the process of refinement of IgG. Separation of the IgA from the other components of the heterogenous T-equine globulin and retention of it with the more easily removable IgG is a practical problem which has not yet been solved satisfactorily for mass production.

Parenthetically, the results suggest that distinct and different kinds of antibodies may contribute to the immunosuppressive effect of ALS. For example, the leukoagglutinins and lymphocytotoxins which seemed not to be in the same location, both appeared to be associated with the ability to mitigate graft rejection.

IMMUNOSUPPRESSION AND ONCOGENESIS

There have been several recent reports of malignant neoplasms developing in chronically surviving human recipients of renal homografts (5, 6, 18, 22, 31). In other publications (18, 23) the ways have been discussed in which a number of factors present in the transplant recipient could have contributed to this situation. Furthermore, the connection between immunosuppression and new tumor growth in animals was the subject of several other communications in this Symposium.

The only point which we wish to make is that there is little justification to implicate uniquely any of the special measures of immunosuppression including ALG which are in clinical use at the present time. This can be appreciated by surveying the means used to prevent kidney homograft rejection in the collected cases of malignancy arising de novo.

There have been 15 clinical cases of new tumor growth in renal homograft recipients (Table 3). Six of these were carcinomas and the other 9 were mesenchymal neoplasms of one kind or other, the majority (7 examples) being reticulum cell sarcomas. All 15 of the patients had received azathioprine and prednisone. Three of the 15 had received ALG at some time before the tumor became detectable. A fourth patient treated by Woodruff (31) had radiographic evidence of an intrathoracic mass before the institution of intravenous ALG treatment.

In our own institution there have been 4 patients with neoplasias which were diagnosed for the first time in the posttransplantation period. Two of these recipients were among the first 106 in our series; they were treated at a time when only azathioprine and prednisone were being given. In the next 110 cases, therapy was with azathioprine, prednisone, and horse ALG. There have also been 2 malignancies in this group after follow-ups ranging from 8 months to 3 years. In both the early and latter series, one each of the patients was cured by conventional treatment.

Although the follow-up is greater in the first than in the second series of cases, the actual incidence will probably remain equivalent since most of the malignant neoplasms thus far collected have made their appearances relatively early after operation. For example, all of the lymphomas have occurred between the 5th and 31st month.

From these findings it is our conclusion that organ transplant recipients have a significantly increased risk of developing a variety of new tumors which are most apt to be of mesenchymal origin. Insofar as can be told, the hazard is a general one and not one which is specifically or uniquely related to any special form of immunosuppressive treatment.

REFERENCES

1. ALLEN, P. Z., S. SIRISINHA AND J. H. VAUGHAN. *J. Immunol.* 95: 918, 1965.
2. ASAKURA, R., AND A. E. REIF. *Cancer Res.* 28: 707, 1968.
3. CARRAZ, M., J. TRAEGER, D. FRIES, J. PERRIN, E. SAUBIER, J. BROCHIER, C. VEYSSEYRE, J. PREVOT, P. BRYON, A. JOUVENCEAUX, J. P. ARCHIMBAUD, P. BONNET, Y. MANUEL, J. P. BERNHARDT AND Y. TRAEGER. *Rev. Inst. Pasteur Lyon* 1: 17, 1967.
4. CLUNIE, G. J. A., B. NOLAN, K. JAMES, J. G. WATT AND M. F. A. WOODRUFF. *Transplantation* 6: 459, 1968.

5. DEODHAR, S. D., A. G. KUKLINCA, D. G. VIDT, A. L. ROBERTSON AND J. B. HAZARD. *New Engl. J. Med.* 280: 1104, 1969.
6. GOOD, F. A. In: *Immunopathology*. Fifth Intern. Symp., edited by P. A. Miescher and P. Grabar. New York: Grune & Stratton, 1967, p. 366.
7. HUME, D. M., J. LEO, R. T. ROLLEY AND G. M. WILLIAMS. In: *Transplantation Proceedings*. New York: Henry M. Stratton, 1969, vol. 1, no. 1, pt. 1, p. 171. (Proc. of 2nd Intern. Congr. of Transp. Soc., 1968.)
8. IWASAKI, Y., K. A. PORTER, J. AMEND, T. L. MARCHIORO, V. ZUHLKE AND T. E. STARZL. *Surg. Gynecol. Obstet.* 124: 1, 1967.
9. JAMES, K. *Clin. Exptl. Immunol.* 2: 615, 1967.
10. JAMES, K., AND P. B. MEDAWAR. *Nature* 214: 1052, 1967.
11. KASHIWAGI, N., C. O. BRANTIGAN, L. BRETTSCHNEIDER, C. G. GROTH AND T. E. STARZL. *Ann. Internal Med.* 68: 275, 1968.
12. KASHIWAGI, N., D. SHERER, C. M. TOWNSEND, R. JACOBS, K. ONO, B. KAPUR AND T. E. STARZL. *Surgery*. In press.
13. MANDEL, M. A., AND R. ASOFSKY. *J. Immunol.* 100: 1259, 1968.
14. MONACO, A. P., M. L. WOOD, J. G. GRAY AND P. S. RUSSELL. *J. Immunol.* 96: 229, 1966.
15. MONACO, A. P., M. L. WOOD, B. A. VAN DER WERF AND P. S. RUSSELL. In: *Antilymphocytic Serum, Ciba Found. Study Group* 29, 1967, p. 111.
16. NAGAYA, H., AND H. O. SIEKER. *Science* 150: 1181, 1965.
17. ONO, K., P. R. F. BELL, N. KASHIWAGI AND T. E. STARZL. *Surgery*, 66: 698, 1969.
18. PENN, I., W. HAMMOND, L. BRETTSCHNEIDER AND T. E. STARZL. In: *Transplantation Proceedings*. New York: Henry M. Stratton, 1969, vol. 1, no. 1, pt. 1, p. 106. (Proc. of 2nd Intern. Congr. of Transp. Soc., 1968.)
19. PICHLMAYR, R., W. BRENDEL, P. H. MIKAELOFF, B. WIEBECKE, J. P. RASSAT, I. PICHLMAYR, J. BOMEL, A. FATCH-MOGHADAM, S. THIERFELDER, K. MESSMER, J. DESCOTES AND M. KNEDEL. In: *Advance in Transplantation*, edited by J. Dausset, J. Hamburger, and G. Mathe. Baltimore: Williams & Wilkins Co., 1968, p. 147. (Proc. of 1st Intern. Congr. of Transp. Soc., 1967.)
20. PICHLMAYR, R., W. BRENDEL, A. TSIRIMBAS, E. BOCK, S. THIERFELDER, A. FATCH-MOGHADAM, B. HORNUNG AND H. PFISTERER. VIII Congr. of Intern. Cardiovascular Soc. *J. Cardiovascular Surg.* 9: 57, 1968.
21. RUSSELL, P. S. *Ann. Internal Med.* 68: 483, 1968.
22. SIEGEL, J. H., R. JANIS, J. C. ALPER, H. SCHUTTE, L. ROBBINS AND M. D. BLAUFOX. *J. Am. Med. Assoc.* 207: 1493, 1969.
23. STARZL, T. E. (with the assistance of C. W. Putnam). *Experience in Hepatic Transplantation*. Philadelphia: Saunders, 1969.
24. STARZL, T. E., L. BRETTSCHNEIDER, I. PENN, R. W. SCHMIDT, P. BELL, N. KASHIWAGI, C. M. TOWNSEND AND C. W. PUTNAM. In: *Transplantation Proceedings*. New York: Henry M. Stratton, 1969, vol. 1, no. 1, pt. 2, p. 448. (Proc. of 2nd Intern. Congr. of Trans. Soc., 1968.)
25. STARZL, T. E., C. G. GROTH, P. I. TERASAKI, C. W. PUTNAM, L. BRETTSCHNEIDER AND T. L. MARCHIORO. *Surg. Gynecol. Obstet.* 126: 1023, 1968.
26. STARZL, T. E., T. L. MARCHIORO AND Y. IWASAKI. *Federation Proc.* 26: 944, 1967.
27. STARZL, T. E., T. L. MARCHIORO, K. A. PORTER, Y. IWASAKI AND C. J. CERILLI. *Surg. Gynecol. Obstet.* 124: 301, 1967.
28. STARZL, T. E., K. A. PORTER, Y. IWASAKI, T. L. MARCHIORO AND N. KASHIWAGI. In: *Antilymphocytic Serum, Ciba Found. Study Group* 29, 1967, p. 4.
29. THIERFELDER, S., D. MOLLER, M. FULITZ, P. DORMER AND I. KIMURA. *Klin. Wochschr.* 46: 277, 1968.
30. WOLSTENHOLME, G. E. W. In: *Antilymphocytic Serum, Ciba Found. Study Group* 29, 1967, p. 146.
31. WOODRUFF, M. F. A., B. NOLAN, J. S. ROBSON AND M. K. MACDONALD. *Lancet* 1: 6, 1969.