Effective and Ineffective Antilymphocyte Sera Correlation of *in Vitro* Tests with Potency of Early Antisera[1] (34584)

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Since there is no reliable *in vitro* assay of the immunosuppressive potency of antilymphocyte serum (ALS), and since preparations of ALS often vary unpredictably in their effectiveness, the clinical use of this putatively valuable agent has proceeded in the face of uncertainty as to the efficacy of the various preparations used. While there is general agreement that lymphocyte agglutination and cytotoxicity titers of potent antisera are elevated, not all antisera with elevated titers are potent. We have investigated this problem in an animal system and have found an immunization schedule which reproducibly results in highly effective rabbit anti-mouse ALS. We have determined that under these conditions the *in vitro* thymocyte agglutination titer reliably predicts *in vivo* immunosuppressive potency. In addition, we have extended our prior observation (1) that repeated immunization of rabbits with mouse lymphocytes, after ALS of optimum potency has been obtained, results in high-titer antisera which have little immunosuppressive effect, and have observed that *in vitro* stimulation of lymphocytes by ALS does not correlate with immunosuppressive potency.

Materials and Methods. Adult New Zealand white rabbits were immunized by injection into the foot pads of 5×10^8 mouse thymocytes or spleen cells in Freund's complete adjuvant. Booster injections of 5×10^8 cells were given intravenously beginning 2 weeks after the initial injection. Antisera were harvested 1 week after completion of immunization and were heated to 56° for 30 min and stored at −20°. No absorption with mouse erythrocytes was done. Immunosuppressive activity was measured by increased survival of C57Bl/6J skin allografts on C3H/HeJ mice injected subcutaneously with ALS. The skin grafts were applied by the technique of Billingham and Silvers (2). After Day 7 grafts were inspected daily for viability, and the day of rejection was taken to be the first day on which total destruction of the graft was evident. There was no gross evidence of toxicity of any of the antisera tested in the doses employed in these studies.

Thymocyte and spleen-cell agglutination titers were performed by incubating serial dilutions of the antiserum to be tested with suspensions of spleen or thymus cells in phosphate-buffered saline (20×10^6 cells per ml) in test tubes at 37° for 30 min. Dilutions of normal rabbit serum and ALS of known high titer were used as controls. After mixing, a large drop of the incubation mixture was transferred to a glass slide, and the degree of agglutination was determined by microscopic observation. The agglutination titer was defined as the maximum dilution which produced agglutination when compared with similarly diluted normal rabbit serum.

Lymphoid cell stimulation was determined by the rate of ^{14}C-L-leucine incorporation into protein by mouse spleen cells or mouse thymocytes cultured in the presence of a 1:80 dilution of ALS. Phytohemagglutinin (PHA-M, Difco)-stimulation of the same preparation of lymphocytes was determined in each case. Normal rabbit serum controls were also performed. The lymphoid cells were incubated for 4 days at 37° in a 5% CO_2 95% air, water-saturated atmosphere at a concentration of 1×10^7 cells per ml in Eagle's medium with 15% fetal calf serum,

[1] Supported by Grants AI 08579 and AM 10824 from PHS and U.S. Army Contract DA-49-193-MD-2621.

TABLE I. *In Vivo* and *in Vitro* Effects of ALS.

Serum	Immunizing cells	No. of immunizing injections	No. mice tested	Mouse skin allograft survival (days)		Thymocyte-agglutination titer ($1/\log_2$)
				Range	Median ± SD	
NRS[a]	—	—	79	8–14	9.8 ± 1.2	1
ALS-5	Spleen	1	9	9–11	9.0 ± 1.1	3
ALS-1	Spleen	3	5	10–13	10.5 ± 1.2	5
ALS-3	Thymus	1	8	10–14	11.4 ± 1.1	5
ALS-6	Spleen	2	9	11–15	12.3 ± 1.1	7
ALS-4	Thymus	2	6	35–51	36.0 ± 1.1	8
ALS-2	Thymus	2	7	24–52	36.0 ± 1.5	9

[a] Normal rabbit serum.

200 U penicillin, 200 μg of streptomycin, and 0.5 μCi of ^{14}C-L-leucine. Protein was isolated, and the radioactivity was counted as described previously (3). The lymphocyte-stimulation index was calculated by dividing the mean counts for three stimulated cultures by the mean counts obtained for cells cultured with normal rabbit serum.

Results. In the first study six antisera were prepared. ALS-1 was harvested after primary immunization of 12 rabbits with spleen cells in adjuvant followed by two booster injections of spleen cells. ALS-2 was obtained by primary immunization of nine rabbits with thymocytes in adjuvant followed by a single booster injection of thymocytes. ALS-3 was harvested from a group of 10 rabbits after primary immunization with thymocytes in adjuvant, and ALS-4 was obtained from the same rabbits after the first booster injection of thymocytes. ALS-5 was harvested from a group of 10 rabbits after primary immunization with spleen cells in adjuvant, and ALS-6 was harvested from the same rabbits after the first booster injection. Allograft survival times listed in Table I were determined in mice that received five injections of 0.1 ml of ALS on alternate days beginning two days prior to grafting. As noted in Table I, the thymocyte-agglutination titers of the sera raised in this study correlated well with the allograft survival times ($r=0.81$, $p<.01$).

In the second study a group of 10 rabbits was initially injected with thymocytes in adjuvant. This was followed by a series of four intravenous booster injections of 5×10^8 thymocytes given at 2-week intervals. Antiserum was obtained from these rabbits 1 week after each injection. The thymocyte-agglutination titers of each of these ALS preparations and the skin-allograft survival times after an injection of 0.1 ml of ALS 2 days before grafting are illustrated in Fig. 1. It is clear that the most effective antiserum was that obtained after the first booster injection (ALS-B). Repeated immunization resulted in progressively less immunosuppressive antisera (ALS-C to ALS-E). After the fifth injection the antiserum (ALS-E) was no more effective than normal rabbit serum. The thymocyte-agglutination titer reached a maximum of 1:1024 after the first booster injection at the same time that the ALS became maximally immunosuppressive (ALS-B). However, the thymocyte-agglutination titer remained elevated after repeated booster immunizations. As noted in Fig. 1, the lymphocyte-stimulation indices also increased with successive immunizations and reached their maximum value after the third booster injection.

Since the results of the first two studies had indicated ALS of high immunosuppressive potency could be prepared by initial immunization with thymocytes in adjuvant followed by a single intravenous booster injection of the same cells, a third study was performed to check the consistency of this finding. Each of 11 rabbits was given an initial immunization and a single booster injection of thymocytes. Antiserum was harvested from each rabbit 1 week after the

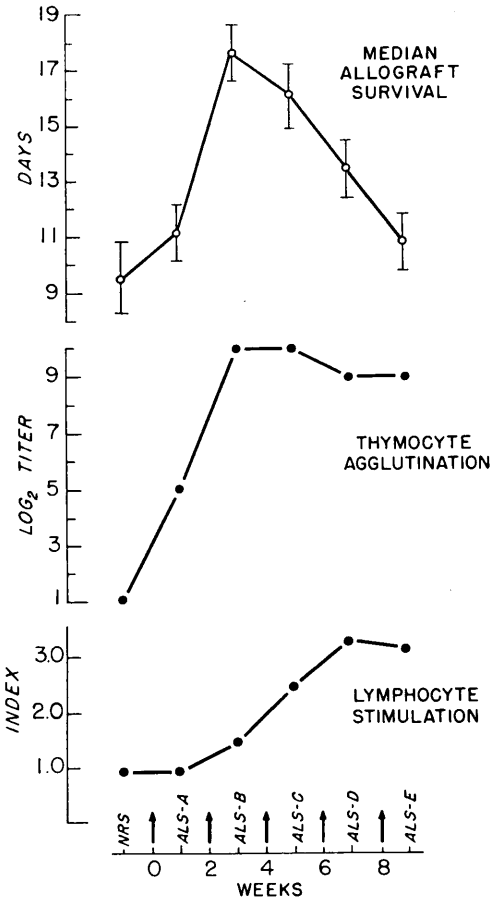

FIG. 1. Median skin allograft survival (brackets indicate 95% confidence limits, thymocyte agglutination titer, and *in vitro* lymphocyte-stimulation index (see text) for normal rabbit serum (NRS) and ALS preparations A-E harvested from the same group of rabbits after successive 2-week immunizations with mouse thymocytes. Arrows indicate times of immunizations

booster injection. The thymocyte agglutination titer and the immunosuppressive potency of each antiserum (given as a single 0.1-ml injection 2 days before grafting) were then tested as noted in Table II. It is apparent that each rabbit yielded antiserum of high immunosuppressive potency after the first booster injection and that the thymocyte agglutination titers of each of these antisera were consistently high.

Discussion. These results indicate that rabbit ALS of high immunosuppressive potency may be regularly produced by an initial injection of thymocytes in adjuvant followed by a single intravenous booster injection of the same cells. In addition, these studies are consonant with the work of others which has suggested that thymocytes yield more potent ALS than spleen cells (4).

Moreover, the present results demonstrate that after repeated immunizations the thymocyte agglutination titer of ALS remains high, but the immunosuppressive potency progressively declines. A similar decline in potency of ALS after increasing numbers of immunizing injections has been observed by Medawar and his associates (5, 6) in some but not all of their studies with rabbit anti-mouse ALS. The same phenomenon was also noted incidentally by Jeejeebhoy and Vela-Martinez (7) who utilized this information to document the fact that leukoagglutination or cytotoxicity titers of ALS were not necessarily correlated with immunosuppressive potency. The present results, on the other hand, indicate that if ALS is harvested from rabbits at the appropriate time, *e.g.*, after the first booster immunization, the thymocyte-agglutination titer does appear to correlate with immunosuppressive potency.

Levey and Medawar (6) concluded that the most likely mechanism of the immunosuppressive action of ALS was the "sterile activation of lymphoid cells." They, therefore, suggested that "the most appropriate *in vitro* test for the potency of a sample of ALS would be one based on its power to produce blast formation." In the present study we have investigated this possibility in a systematic fashion and have found that *in vitro* stimulation of lymphocytes by ALS did not correlate with the immunosuppressive potency of preparations tested. In fact, the *in vitro* stimulating capacity of ALS increased with increasing numbers of booster injections while the immunosuppressive potency of the same ALS preparations was progressively declining.

In view of the present finding that, in the rabbit at least, multiple booster injections lead to a progressive decline in the potency of ALS, it is of interest to note that several of the preparations of ALS currently in clinical

TABLE II. *In Vivo* and *in Vitro* Effects of ALS.

No. of rabbit source of ALS	No. mice tested	Mouse skin allograft survival (days)		Thymocyte-agglutination titer ($1/\log_2$)
		Range	Median ± SD	
NRS[a]	79	8–14	9.8 ± 1.2	1
697	6	13–38	18.5 ± 1.4	10
163	5	15–22	17.5 ± 1.2	8
335	6	13–22	15.9 ± 1.4	8
769	7	14–22	16.3 ± 1.2	9
961	9	10–23	16.2 ± 1.1	9
298	8	12–20	16.2 ± 1.3	8
25	8	13–21	15.2 ± 1.2	9
842	8	10–23	15.3 ± 1.4	9
725	8	14–20	15.6 ± 1.2	10
742	8	15–20	15.0 ± 1.2	8
767	7	13–18	15.5 ± 1.2	9

[a] Normal rabbit serum.

use have been harvested from horses that have received multiple booster injections of human lymphoid tissue (8, 9). While it is true that ALS of demonstrable immunosuppressive activity has been raised in horses which received multiple booster injections of dog lymphocytes (10, 11), it would appear desirable to investigate the possibility that at some point increasing immunization of the horse, like the rabbit, may yield ALS of diminished potency.

Summary. The thymocyte-agglutination titer of ALS raised in rabbits against mouse lymphoid cells correlated with immunosuppressive potency of antisera harvested after one or two immunizing injections. However, when ALS preparations were harvested from rabbits given multiple booster injections of lymphocytes the thymocyte-agglutination titer remained elevated whereas immunosuppressive potency progressively declined. The capacity of ALS preparations to stimulate mouse lymphocytes *in vitro* did not correlate with the immunosuppressive potency of these antisera.

The technical assistance of J. Parker and M. Langley is gratefully acknowledged.

1. Davis, R. C., Cooperband, S. R., and Mannick, J. A., Surgery **66**, 58 (1969).

2. Billingham, R. E., *in* "Transplantation of Tissues and Cells." Wistar Institute Press, Philadelphia (1961).

3. Cooperband, S. R., Bondevik, H., Schmid, K., and Mannick, J. A., Science **159**, 1243 (1968).

4. Greaves, M. F., Tursi, A., Playfair, J. H. L., Torrigiani, G., Zamir, R., and Roitt, I. M., Lancet **1**, 68 (1969).

5. Jooste, S. V., Lance, E. M., Levey, R. H., Medawar, P. B., Ruszkiewicz, M., Sharman, R., and Taub, R. N., Immunology **15**, 697 (1968).

6. Levey, R. H., and Medawar, P. B., Proc. Nat. Acad. Sci. **56**, 1130 (1966).

7. Jeejeebhoy, H. F., and Vela-Martinez, J. M., Transplantation **6**, 149 (1968).

8. Pichlmayr, R., Brendel, W., and Zenker, R., Surgery **61**, 774 (1967).

9. Starzl, T. E., Groth, C. G., Terasaki, P. I., Putman, C. W., Brettschneider, L., and Marchioro, T. L., Surg. Gynec. Obst. **126**, 1023 (1968).

10. Abaza, J. M., Nolan, B., Watt, J. G., and Woodruff, M. F. A., Transplantation **4**, 618 (1966).

11. Monaco, A. P., Abbott, W. M., Othersen, H. B., Simmons, R. L., Wood, M. L., Flax, M. H., and Russell, P. S., Science **153**, 1264 (1966).

Received Nov. 3, 1969. P.S.E.B.M., 1970, Vol. 133.