# Immunosuppressive Properties of Heterologous Antilymphocytic Sera Prepared Against Thymocyte Soluble Fraction[1] (36641)

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(Introduced by D. G. Scarpelli)

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Antilymphocytic sera (ALS) and antilymphocytic globulin (ALG) have been shown to be potent and effective immunosuppressive agents. The properties and effects of various ALS preparations, both *in vivo* and *in vitro*, have been intensively studied (1, 2). Heterologous antilymphocytic sera (ALS) are usually prepared against whole cells from lymphoid tissues. Recently, however, potent antisera have been produced against subcellular fractions of mouse, rat and human lymphoid tissues (3–10). In the present study, mouse thymus soluble fractions of varying immunogenicity have been used to produce antilymphocytic sera. These antisera markedly prolonged skin allograft survival times and suppressed antibody production to sheep erythrocytes in mice, thus proving to be potent suppressors of both cellular and humoral immunity. The present report describing solubilization of thymocyte antigens is a part of a continuing study of those antigens which are capable of evoking the production of immunosuppressive antilymphocytic antibodies.

*Materials and Methods. Animals.* Male C57Bl/6J, CBA and BALB/c mice, 18–20 g in weight, were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine. New Zealand white rabbits (4–5 kg body weight) were used for preparation of antilymphocytic sera.

*Preparation of thymus soluble fractions.* Thymus cells which had been obtained from C57B1/6J mice were suspended in sucrose buffer pH 7.5 (0.25 *M* sucrose, 0.05 *M* Tris, 0.025 *M* KCl, 0.005 *M* $MgCl_2 \cdot 6\ H_2O$). The cells were washed once in buffer solution and then allowed to stand for 15 min at 4°. The washed cell suspension was homogenized in a Potter–Elvehjem tissue grinder with a teflon plunger for 30–45 min at 4°, following which 90–95% of the cells were disrupted. The homogenates were sedimented at 105,000*g* in an International Ultracentrifuge (Model B 60) either for 30 or 60 min at 4°. The cell-free, clear supernatants (soluble fractions), designated $S_6$, $S_7$, $S_9$, and $S_{10}$, prepared as indicated below, were separated and then used for immunization either immediately or one month following storage at —20°.

($S_6$) Cells homogenized, spun for 60 min, sediment discarded, and supernatant frozen, thawed and then used for immunization. ($S_7$) Cells not homogenized, but shaken in sucrose buffer pH 7.5 for 30–45 min, spun for 60 min, sediment discarded, and supernatant frozen, thawed and then used for immunization. ($S_9$) Cell homogenate spun for 30 min, sediment discarded, and supernatant immediately used for immunization. ($S_{10}$) Homogenate spun for 30 min, sediment discarded, and supernatant frozen, thawed and then used for immunization.

*Immunization.* On Day 0 rabbits were injected into their front and hind footpads with soluble fraction preparations, either fresh or frozen-thawed, which had been emulsified with an equal volume of Freund's complete adjuvant. Each animal received the soluble preparation from $6 \times 10^8$ thymus cells. Two weeks later they were given an intravenous

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booster injection of soluble fraction without adjuvant, and one week following this the animals were heparinized (100 U iv) and bled out by cardiac puncture. Serum was separated, divided into 2 ml aliquots, labelled according to the fraction used for immunization ($S_6$–$S_{10}$) and then stored at —20°.

*Skin grafting.* The ability of these heterologous antilymphocytic sera, prepared against thymus soluble fractions, to prolong skin allograft survival was determined. Donor skin approximately 1 cm in diameter was taken from the abdomen of male CBA mice and placed on beds prepared on the right thoracic wall of male C57Bl/6J recipients. Each graft was sprayed with Aeroplast, covered with gauze, and secured in place with a Band-Aid (Johnson and Johnson). Groups of male C57Bl/6J mice, each consisting of eight animals, were grafted with skin from male CBA donors on Day 0. Mice were given 0.5 ml of ALS sc on Days +2 and +5 post grafting. Bandages were removed on Day 9, and then grafts examined visually every day until completely rejected.

*Leukoagglutinin and cytotoxic antibody titrations.* Agglutinating and cytotoxic antibodies directed against mouse lymphocytes were determined according to the method of Jeejeebhoy (11). Thymus cells were washed twice in phosphate buffered saline (PBS, pH 7.2), resuspended in PBS with 10% heat inactivated syngeneic mouse serum and final concentration adjusted to $5 \times 10^6$ cells/ml. One tenth ml of cell suspension was incubated with 0.1 ml of doubling dilutions of antilymphocytic sera for 1 hr at 37°. Following this, 0.1 ml of undiluted reconstituted guinea pig serum was added, and the cells incubated for another 30 min at 37°. Living and dead cells were differentiated by staining with 0.1% of freshly prepared trypan blue. Agglutination and cytotoxic titers were determined simultaneously.

*Immune response to sheep erythrocytes.* BALB/c mice were injected intraperitoneally (ip) with 0.25 ml of either antisera $S_6$–$S_{10}$ or normal rabbit serum (NRS) on Days —1, 0, and +1. They were immunized with a single ip injection of 2% sheep erythrocytes ($4 \times 10^8$ cells) on Day 0. Four days later splenic plaque-forming cells (PFC) were enumerated according to the method of Jerne *et al.* (12), as modified by Mishell and Dutton (13) using glass slides.

*Hemagglutination titrations.* Equal volumes of 0.8% suspension of SRBC (0.05 ml) and serial twofold dilutions of serum in PBS were added to Microtiter plates and allowed to incubate for 1 hr at 37°. Hemagglutination was scored from 3+ to 0.

*Hemolysin titrations.* Equal volumes of 0.8% suspension of SRBC (0.05 ml) and doubling dilutions of serum in PBS, were added to Microtiter plates and allowed to incubate for 10 min at 37°. Following this, 0.05 ml of a 1:20 dilution of reconstituted guinea pig serum (Hyland Laboratories, California) in Tris-buffered saline (pH 7.4) was added, and the plates were incubated for another hour at 37°. The end point was taken as the last well showing complete hemolysis.

The sheep erythrocytes (SRBC) used for immunization, assessment of PFC and serum titers were obtained from the same animal.

*Statistical methods.* Means and standard errors were determined from pooled data.

*Results.* The leukoagglutinating and cytotoxic titers of antilymphocytic sera prepared against various soluble fractions are given in Table I. All sera except $S_7$ had high titer agglutinating and cytotoxic antibodies directed against mouse lymphocytes. Although there is no good correlation between cytotoxic antibody titers and immunosuppressive potency (1, 2, 10, 11), nevertheless their presence at least indicates that sensitization has occurred.

Skin allograft survival times were prolonged in mice which had been treated with

TABLE I. Agglutination and Cytotoxic Antibody Titers Directed Against Mouse Lymphocytes.

| Serum no. | Reciprocal antibody titer | |
|---|---|---|
| | Agglutination | Cytotoxic |
| $S_6$ | 512 | 512 |
| $S_7$ | <4 | <4 |
| $S_9$ | 512 | 128 |
| $S_{10}$ | 512 | 128 |
| NRS | <4 | <4 |

TABLE II. Survival Time of CBA to C57Bl/6 Skin Allografts on Mice Treated with Antilymphocytic Sera (ALS) Prepared Against Thymus Soluble Fractions.

| Serum[a] | No. of grafts | Range (days) | MST ± SE (days) |
|---|---|---|---|
| $S_6$ | 8 | 13–25 (13, 2 × 15, 3 × 17, 20, 25) | 17.3 ± 3.5 |
| $S_7$ | 8 | All rejected by Day 10 | 10 |
| $S_9$ | 8 | 14–23 (14, 16, 17, 18, 2 × 19, 22, 23) | 18.5 ± 2.8 |
| $S_{10}$ | 8 | 11–27 (11, 14, 16, 18, 21, 24, 27) | 18.7 ± 5.2 |
| NRS | 8 | All rejected by Day 10 | |

[a] Recipient mice were given 0.5 ml of either ALS or normal rabbit serum (NRS) subcutaneously (sc) on Days +2 and +5 post grafting.

ALS $S_6$, $S_9$, and $S_{10}$. Mean survival times ranged from 17.3 to 18.7 days as compared to 10 days for normal serum (NRS) treated mice and those given $S_7$ (Table II). Homogenized soluble preparations of thymocytes $S_6$, $S_9$, and $S_{10}$ elicited the production of immunosuppressive antilymphocytic antibodies, while in contrast the nonhomogenized soluble preparation $S_7$ did not. It is apparent from our data that immunogenic soluble fractions are only obtained when cells are disrupted.

The number of splenic plaque-forming cells (PFC) following immunization with SRBC was markedly depressed in animals treated with antisera $S_6$, $S_9$, and $S_{10}$ as shown in Table III. Animals treated with ALS had 2000–3000 splenic PFC as compared to 50,000–60,000 PFC in mice injected with SRBC alone, normal rabbit serum (NRS), or serum $S_7$.

Serum hemagglutinin and hemolysin titers were correspondingly depressed in ALS treated mice when compared to normal rabbit serum treated controls (Table III).

*Discussion.* The present study shows that antisera prepared against soluble fractions of thymocytes are potent immunosuppressive agents which are capable of prolonging skin allograft survival as well as depressing humoral antibody production to sheep erythrocytes in mice. Cytotoxic and agglutinating antibodies were present in high titer in the three serum pools $S_6$, $S_9$, and $S_{10}$ which had immunosuppressive activity and in very low titer in pool $S_7$ which was not immunosuppressive. Although there is no good correlation between cytotoxic and agglutinating antibody titers and immunosuppressive activity, nevertheless, sera devoid of such antibodies generally have not been reported to be immunosuppressive.

TABLE III. Immunosuppressive Effect of Rabbit Antimouse Lymphocyte Sera (ALS) on the Antibody Response to Sheep Erythrocytes.

| | Splenic plaque-forming cells[b,c] | | $Log_2$ of reciprocal serum titer[b] | |
|---|---|---|---|---|
| Serum treatment[a] | Total | PFC/$10^3$ | HA | Hem |
| None | 57,312 ± 4,168 | 467 ± 51 | 7 | 8.5 |
| Normal rabbit serum | 47,466 ± 5,545 | 366 ± 14 | 6 | 7.5 |
| ALS $S_6$ | 4,870 ± 745 | 78 ± 8 | 3 | 3.3 |
| ALS $S_7$ | 40,277 ± 5,923 | 283 ± 19 | 6.3 | 8 |
| ALS $S_9$ | 2,208 ± 310 | 22 ± 3 | 1.1 | nil |
| ALS $S_{10}$ | 2,161 ± 656 | 23 ± 6 | 1.5 | nil |

[a] Mice were treated with 0.25 ml of ALS or NRS ip on Days −1, 0, and +1 and immunized with 1 ml of 2% SRBC on Day 0.

[b] Splenic PFC and serum hemagglutinin (HA) and hemolysin (Hem) titers were determined 4 days following immunization with SRBC.

[c] The mean and standard error are recorded.

Antisera which have been prepared against thymus soluble fractions were as potent as those which had been raised against intact thymic or lymph node lymphocytes either alone or in combination. Disruption of the cells prior to solubilization was absolutely essential for the release of antigens, as shown by the lack of immunogenicity of preparation $S_7$. Freezing, prolonged storage and thawing of the soluble fractions obtained from disrupted cells did not alter their immunogenicity.

Soluble fractions of lymphocytes from various lymphoid tissues have been used for the production of antilymphocytic sera. Thymus cell fractions have been reported to be the most immunogenic and spleen cell fractions only weakly immunogenic (7, 9, 10). Cell fractionation studies have shown that both thymus membrane preparations and mitochondrial fractions are capable of evoking the production of antilymphocytic antibodies (4, 5), although one study indicated that the mitochondrial fraction produced antisera which were toxic in high doses (4). Antisera prepared against cell sap of lymphocytes have also been reported to have immunosuppressive properties (6, 7). All of these studies, including our own, show that membrane or cell sap preparations are potent immunogens.

Solubilization of antigen may be useful under those circumstances when harvested lymphocytes cannot be used immediately for immunization. Furthermore, purification and immunochemical characterization of solubilized antigens of lymphocytes and thymocytes should eventually allow us to produce potent and highly specific sera which may suppress either cell-mediated immunity, humoral antibody production, or both.

*Summary.* Soluble, cell-free fractions of mouse thymocytes were potent immunogens capable of evoking the production of immunosuppressive antilymphocytic antibodies in rabbits. These sera were capable of prolonging survival times of CBA to C57Bl/6 skin allografts, and depressing humoral antibody production and splenic plaque forming cells in mice immunized with sheep erythrocytes. Disruption of thymus cells prior to solubilization was absolutely essential for the release of antigens. Freezing, prolonged storage and thawing of the soluble fractions obtained from disrupted cells did not alter their immunogenicity. Antisera prepared against thymocyte soluble fractions were equally as immunosuppressive as those prepared against intact cells.

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