Effects of an Antiserum to Calf Thymosin on Lymphoid Cells *in Vitro** (33524)

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Since the first description of the immunological consequences of thymectomy in neonatal mice (1, 2), interest has grown in the possible existence of a cell-free, biologically active, humoral substance of thymic origin. Support for the concept of a thymic humoral factor was derived from the observation that thymic grafts enclosed in Millipore chambers restored immunological competence to neonatally thymectomized mice (3). Subsequently an extract of calf thymus, termed thymosin, was shown to stimulate lymphocytopoiesis and lymphoid tissue growth of mice *in vivo* as reflected by the incorporation of labeled precursors into the DNA and protein of lymphoid tissue (4, 5). This thymosin preparation also effectively restored immunological competence in F_1 hybrid neonatally thymectomized mice as measured by the graft versus host reaction after parenteral injection of spleen cells (6). In addition, it has been demonstrated in these laboratories (7) that daily administration of thymosin to normal adult mice accelerated the rate of rejection of first and second set skin grafts. In contrast, daily injection of an antiserum to calf thymosin prepared in rabbits delayed significantly the rate of rejection of these grafts. These findings, together with the demonstrations of the immunosuppressive effects of antisera against thymocytes (8, 9) and lymphocytes (10–12) led us to investigate further the antigenic properties of thymosin. Specifically, it was of interest to determine whether the cell-free thymic preparation, thymosin, would induce formation of antibodies that would have cytotoxic or agglutinating effects upon lymphoid cells.

Materials and Methods. The thymosin used is an acetone precipitated, relatively heat-stable saline extract of calf thymus that contains several electrophoretically distinct components (5). For purposes of immunization, the thymosin preparation was dissolved in saline (30 mg of protein [Lowry method (13)] per ml,) and 2 ml were emulsified with an equal volume of complete Freund's adjuvant (Difco). This emulsion was injected in four intradermal sites in each of 4 rabbits (white New Zealand, 2.5–3.5 kg). Two weeks later the rabbits each received additional injections of 2.0 ml of thymosin (60 mg of protein), without adjuvant, every other day for 6 days. Seven days after the last injection the rabbits were bled and the sera were separated, pooled, and stored at —20°. This serum will be referred to as antithymosin serum (ATS). Normal rabbit serum (NRS) that was to serve as control was obtained from nonsensitized rabbits and processed in the same manner.

Detection of circulating antibody. The pooled serum (ATS) was examined for antibody to the thymosin preparation by precipitation in agar immunodiffusion plates (Mann Research Laboratories Inc., New York, N. Y.). In each instance the central well was filled with undiluted serum and the six peripheral wells were filled with dilutions of thymosin ranging from 0.15 to 5.0 mg of protein/ml. As control for the antisera, cen-

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ter wells of separate plates received normal rabbit serum instead of the experimental serum. As control for the antigen, bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, Mo.,) and fractions prepared from calf liver, calf spleen, and calf kidney in the same manner as thymosin were used in the peripheral wells. Test plates were kept in a humid environment at room temperature and inspected daily for 12 days; the results generally became apparent at 48–72 hr. The plates were then read and photographed.

Cytotoxicity. Cytotoxic tests were performed as outlined by Gray *et al.* (14). Thymus, lymph nodes, and spleen were obtained from A/Jax mice, white New Zealand rabbits and calves immediately following exanguination. The tissues were cut cleanly and touched repeatedly to two drops of saline in a shallow well on a slide. At least 2×10^6 cells from thymus, lymph nodes, or spleen were suspended in 2 drops of 0.15 *M* NaCl. The cell suspensions were then mixed with an equal volume of either ATS, NRS, or saline and incubated at 37° for 30 min. The viability of cells was determined by adding freshly prepared 0.2% trypan blue to the incubated cell suspensions and counting the stained and unstained cells (a total of 200) in a hemocytometer.

Leukoagglutination. Leukoagglutination studies were conducted essentially as described by Amos and Peacocke (15). Suspensions of cells were prepared from lymph nodes, thymus, and spleen of A/Jax mice, white New Zealand rabbits, and calves by teasing the appropriate organs in Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.). The cells were passed through each of two strainers (mesh nos. 100 and 200, Baruch Instruments Corp., N. Y., N.Y.) and washed three times with 0.15 *M* NaCl by centrifugation. The sedimented cells were then resuspended in a phosphate buffer (15) to a final concentration of $4.5–8.0 \times 10^7$ cells/ml. Serial dilutions of ATS, NRS, and saline were mixed in test tubes with an equal volume (0.05 ml) of cells and the tubes incubated at 37° for 2 hr.

TABLE I. Cytotoxic Effect of a Rabbit Antiserum to Calf Thymosin on Lymphoid Cells from Three Species.

Origin of cells	Dead cells (%) after incubation with: Saline	Normal rabbit serum	Anti-thymosin serum
Calf			
Thymus	12	9	96
Lymph node	15	12	30
Spleen	12	17	44
Mouse (A/Jax)			
Thymus	6	13	77
Lymph node	12	15	21
Spleen	20	21	18
Rabbit (New Zealand white)			
Thymus	16	21	49
Lymph node	20	15	18
Spleen	22	18	15

The suspensions were then gently agitated and read microscopically in a wet preparation for agglutination.

Forssman antibody. Examination of the ATS for the possible presence of heterophile antibodies was conducted by standard clinical procedures (16).

Results. Detection of circulating antibody. Pooled sera from rabbits immunized with the thymosin preparation contained precipitating antibody for at least two of the several protein components present in the thymosin fraction; two lines of precipitation developed on the immunodiffusion plates. One line of precipitation developed identity with BSA as well as with the extracts of calf liver, kidney, and spleen. Absorption of the antiserum with BSA removed the antibody responsible for this precipitin line and left only one precipitin line to another component of the thymosin preparation (Fig. 1). NRS caused no precipitation with any of the extracts.

Cytotoxicity. The cytotoxicity of ATS to lymphoid cells from three species of animals is summarized in Table I. ATS was markedly cytotoxic primarily for the thymic cells of the calf, mouse and rabbit (96, 77, and 49%

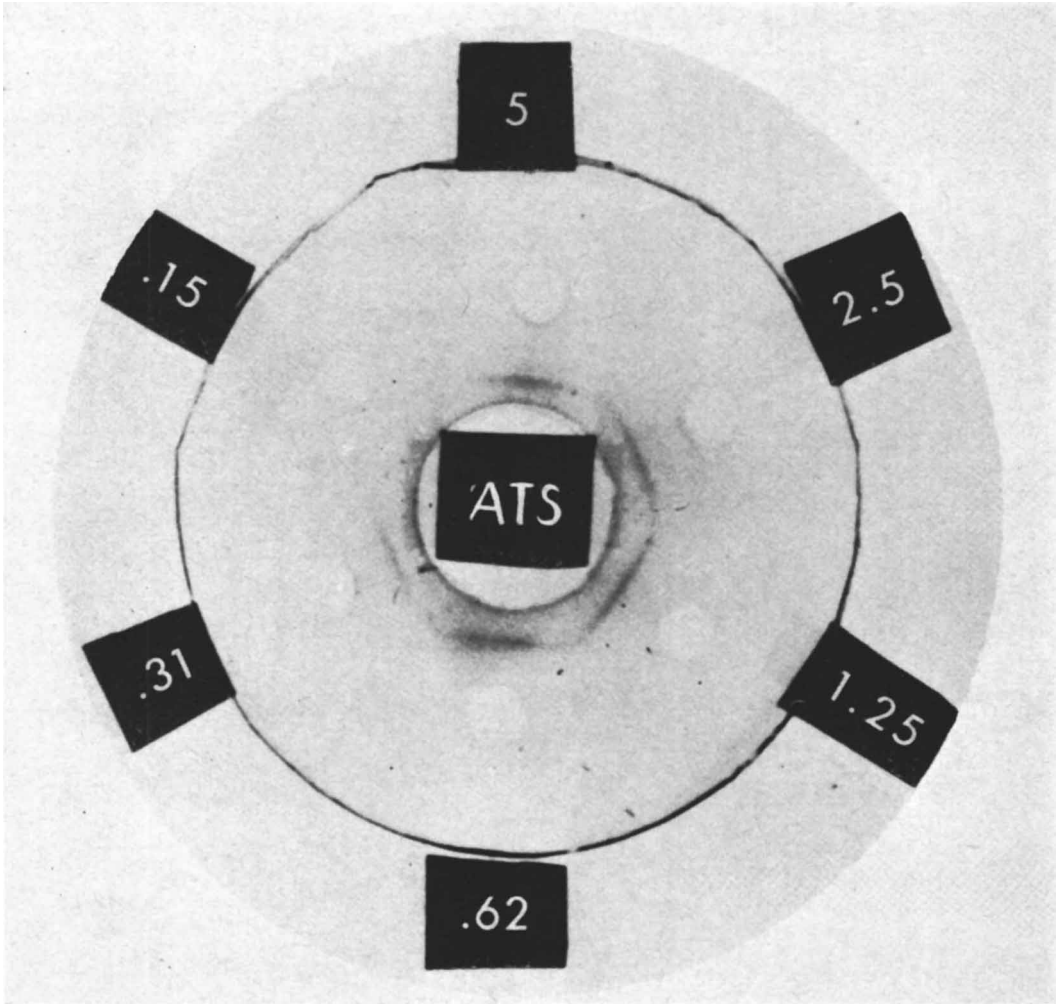

FIG. 1. Precipitin lines resulting from the reaction of various concentrations of calf thymosin (fraction 3) to an antiserum to this fraction prepared in rabbits. Prior to use, the thymosin antiserum (ATS) in the center well had been adsorbed with bovine serum albumin. The concentrations of thymosin in milligrams per milliliter were as indicated by the numbers adjacent to the outside wells.

of the respective cells were dead). Antithymosin serum had no appreciable cytotoxic effect on lymph node cells or spleen cells of mice and rabbits but was weakly cytotoxic to lymph node and spleen cells of calves (30 and 44% of these calf cells were dead).

Leukoagglutination. Table II presents the relative agglutinating activity of rabbit ATS with lymphoid cells from the mouse, rabbit, and calf. The most striking finding was that ATS reacted with thymus cells of all three species, although to different degrees. ATS agglutinated calf thymic cells when diluted to 1:1024, mouse thymic cells at a dilution of 1:256 and rabbit thymic cells at a dilution of 1:32. Calf lymph node cells were agglutinated by ATS diluted to 1:512, while ATS only weakly agglutinated mouse and rabbit lymph node cells and spleen cells.

Detection of Forssman antibody. In order to assess whether the activity of ATS was due to an antibody to a Forssman antigen, the serum was adsorbed with guinea pig kidney antigen and beef red cell antigen (Certified

TABLE II. Agglutination of Thymic, Splenic, and Lymph Node Cells from Three Animal Species by a Rabbit Antiserum to Calf Thymosin.

Origin of cells	Saline	Normal rabbit serum	Anti-thymosin serum[a]
Calf			
Thymus	0	0	1024
Lymph node	0	0	512
Spleen	0	0	4
Mouse (A/Jax)			
Thymus	0	0	256
Lymph node	0	0	2
Spleen	0	0	32
Rabbit (New Zealand white)			
Thymus	0	0	32
Lymph node	0	0	8
Spleen	0	0	4

[a] Reciprocal of highest dilution causing agglutination.

Blood Donor Service Inc., Jamaica, N. Y.) and the cytotoxicity and leukoagglutination reactions were repeated using the adsorbed ATS. The unadsorbed sera did not agglutinate sheep erythrocytes. In addition, no difference was detected between the unadsorbed serum and the serum adsorbed with guinea pig kidney and that adsorbed with beef erythrocytes in the cytotoxicity and leukoagglutination reactions with the lymphoid cells of the three species tested. Thus, the activity of ATS was not attributable to antibody directed to a Forssman antigen.

Discussion. The wide interest in the role of the thymus in immunological phenomena, particularly those related to cell-mediated responses, has led to investigations of various lymphocyte-stimulating thymic extracts (4, 5, 17–19). The preparation utilized in the present study as an antigen for the production of antiserum is a cell-free, soluble, partially purified fraction of calf thymus and has been termed thymosin (5). When this antiserum was diffused in agar-gel against thymosin, BSA, and fractions prepared from calf liver, kidney, and spleen, a precipitin line of identity formed to all substances. In addition, a second precipitate formed between ATS and thymosin alone. When ATS was adsorbed with BSA, the precipitin line against BSA and extracts of calf liver, kidney, and spleen no longer appeared. The adsorbed ATS still produced one precipitin line against thymosin. Thus, ATS contained at least two antibodies, one precipitated with the partially purified thymosin and the other precipitated with BSA and extracts of calf liver, kidney, and spleen.

Unadsorbed and adsorbed ATS exhibited the same degree of cytotoxic and agglutinating activity to thymocytes of the three species tested, namely, mouse, rabbit, and calf. Furthermore, when ATS was added to lymphocytes, it had cytotoxic and agglutinating activity against calf lymphocytes but not against lymphocytes of the other species tested. This suggests that thymosin, a cell-free extract, contains a soluble antigen common to cell surfaces of thymocytes. This soluble antigen appears to be common qualitatively, if not quantitatively, to the three species studied. The most striking results were obtained when thymocytes from the calf were used; this was not unexpected since the antiserum was prepared against an extract of calf thymus. Previous studies of antilymphocyte and antithymocyte sera have demonstrated that these sera were toxic only for the cells of the species from which the antigen was derived, and only rarely were toxic for cells of different strains of the same species (12). The fact that a cell-free preparation rather than whole cells was used for immunization in these studies may explain why our ATS had cytotoxic and agglutinating activity when tested with thymocytes of species other than the calf.

Inasmuch as the ATS was cytotoxic for the thymus cells of the calf, mouse, and rabbit and agglutinated the thymus and lymph node cells of the calf, it was surprising to find the weak or absent agglutination of lymphocytes of the mouse and rabbit by ATS. It is possible that calf lymphocytes share an antigen with calf thymocytes which is species specific and may differ from another antigen that is common to cell surfaces of thymocytes of

several species. This would suggest that the calf thymosin preparation not only contains an antigen common to thymocytes but also a species-specific antigen present on cell surfaces of calf thymocytes. That this antigen is not a Forssman hapten was indicated by the fact that ATS did not agglutinate sheep erythrocytes and when adsorbed with guinea pig kidney, it continued to show the same cytotoxic and leukoagglutination reactions as before adsorption. Alternatively, the data suggest that calf lymph nodes may be populated by cells that originate from the thymus and only one cellular antigen is present in the thymosin preparation used.

The results of the present study indicate that a cell-free, soluble thymic antigen can give rise to an antiserum which has cytotoxic and agglutinating activity against thymocytes. It should be stressed that this antigen appears to be common to several species. That the same antiserum strongly agglutinates lymphocytes only of the species from which the antigen originated lends support to the concept of an antigenic distinction between thymocytes and lymph node cells (20, 21). The possible value in immunosuppression of an antiserum to a soluble antigen present in the thymus and apparently present on cell surfaces of thymocytes is being currently studied in skin allograft models. Our present data indicate that daily injections of a rabbit antiserum to calf thymosin significantly prolongs the survival of first and second set skin allografts from C57BL/6 mice transplanted to A/Jax mice (7). A lymphopenia was not evident in the mice that showed prolonged skin-allograft survival. This implies that one of the antigens in thymosin that appears to be common to cell surfaces of thymocytes may be a significant factor in skin-allograft rejection, and that it may be inhibited, in a still unexplained manner, by an antithymosin serum.

Summary. A lymphocytopoietic fraction of calf thymic tissue, thymosin, has been utilized to prepare an antiserum in rabbits. The antiserum contained antibodies to at least two of the several components of the thymosin fraction. One of these precipitable antibodies was shown to be specific for a constituent of the thymosin preparation; the other was directed to bovine serum albumin. The antithymosin serum, after removal of antibody to BSA, was markedly cytotoxic to thymus cells but not to lymph node or spleen cells from calves, mice, and rabbits. The antithymosin serum agglutinated thymus cells of calves and cross-reacted with thymus cells of mice and rabbits. These activities of the antiserum to thymosin were not due to a Forssman antigen. The results support the concept of an antigenic difference between thymocytes and lymph node cells and provide evidence for the presence of a cell-free, soluble thymic antigen in thymosin.

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Urine Solute Composition of Rats Exposed to Chronic Centrifugation* (33525)

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Chronic exposure of rats to simulated high gravity can cause an increased urine output (1). At 1.7*g* and 3.0*g*, the urine volumes were found to increase in 4–6 days by 200–300% and then drop back towards control levels. Furthermore, while an initial decrease in water consumption was followed by an increase and probable overshoot, such increases did not match those measured for the urine volumes. The present paper is part of a continuing attempt to understand the influence of the inertial field environment upon fluid balance. Since there are two general mechanisms whereby urine flow can be increased (2), namely, osmotic diuresis and water diuresis, the information on urine solute composition described here was obtained in order to gain some insight into the etiology of the observed centrifugation polyuria.

Methods and Materials. Throughout the preexposure, experimental and control conditions, male Simonsen rats (250–350 g) were housed two to a metabolism cage fitted with a drinking device and an internal feeder containing ground Purina lab chow. The general procedures relating to animal maintenance and to 24-hr measurements for body weight, food and water intakes, and urine output for both the baseline and exposure periods were essentially the same as those previously described (1). After at least 1 week of preconditioning to handling and to the room environment and after baseline measurements, a total of 36 unrestrained rats, constituting two separate studies, were transferred to a 205-cm radius centrifuge (3) and exposed to a resultant inertial field of either 3.0*g* or 1.7*g* at 36.1 rpm. The centrifuge was run continuously except for daily stoppages of approximately 15 min for measurements and for cage maintenance. Thirty-two control animals were housed in cages postioned along the periphery of the centrifuge enclosure where environmental conditions, with the exception of the inertial field, were similar to those for the experimental animals.

Urine osmolalities (mosmols/kg of water) for all animals were measured without dilution on the day of collection by a freezing point depression method in which sodium chloride solutions of known osmotic concentration were employed as the standards of comparison. The total, urinary, osmotically-effective, excretion load (mosmols/day; hereafter referred to as total solute excretion) was calculated as the urine volume multiplied by the urine osmolality.

PART I (3.0*g*). Six rats were centrifuged for 17 days to study the effects of exposure to 3.0*g* upon urine osmolality and calculated total solute excretion. These results were compared with those from 4 *ad libitum* fed control animals (subsequently to be referred to as *ad libitum* control animals).

PART II (1.7*g*). In order to determine the

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