*EFFECT OF THYMOSIN AND AN ANTITHYMOSIN SERUM ON ALLOGRAFT SURVIVAL IN MICE**

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The isolation and partial purification from calf thymus of a soluble lymphocytopoietic factor termed thymosin has been reported.[1] When administered to normal mice, this factor stimulated lymphocytopoiesis as reflected by an increase in peripheral lymphoid tissue weight and augmented incorporation of labeled precursors into the DNA and protein of lymphoid cells.[1-3] Thymosin also accelerated lymphoid tissue regeneration in mice exposed to whole-body X irradiation,[4] restored immunological competence to spleen cells from neonatally thymectomized mice as measured by a graft-versus-host response assay,[5] and reduced the incidence of wasting and death in neonatally thymectomized mice.[6] In studies with lethally irradiated, thymectomized mice injected with syngeneic bone marrow cells, it has been found that thymosin increased the number of plaque-forming cells per spleen.[7]

In view of the previously demonstrated immunosuppressive properties of antithymocyte sera,[8, 9] experiments were designed to examine the effect on skin allograft survival in mice of a thymosin fraction and an antiserum prepared to this soluble fraction of calf thymus.

Materials and Methods.—Preparation of thymosin and of antithymosin serum: Thymosin was prepared from calf thymus according to the method of Goldstein, Slater, and White;[1] the acetone-insoluble, relatively heat-stable fraction (fraction 3) was used in these experiments. The antithymosin serum (ATS) was prepared in male white New Zealand rabbits (2.5–3.5 kg). The thymosin fraction was dissolved in saline in a concentration of 30 mg protein/ml; protein analyses were done by the Lowry method.[10] Two milliliters of the thymosin solution were emulsified with an equal volume of complete Freund's adjuvant (Difco) and injected intradermally in four sites into each rabbit. After 2 weeks, additional injections of 2 ml of thymosin (60 mg protein) without adjuvant were given subcutaneously to each rabbit every other day for 6 days. The animals were bled 1 week after the last injection. All the sera were pooled and stored at −20°C. Normal rabbit sera (NRS) were obtained from nonsensitized rabbits.

Allograft procedures; effect of thymosin: The effect of thymosin on skin allograft survival was studied in 8–9-week-old male $B_{10}D_2/Sn$ mice receiving skin from C57BL/6 mice. The recipients were divided into six groups of 10–15 mice each. One week prior to grafting and throughout the period of the experiment, three experimental groups received daily subcutaneous injections of 0.5, 1.0, and 4.0 mg of thymosin, respectively. Three control groups received either 2.0 mg of calf liver extract (prepared in the same manner as thymosin), 4.0 mg bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, Mo.), or saline. Circular skin grafts (1.0 cm) were performed according to the method of Billingham and Medawar.[11] The grafts were evaluated daily by visual and tactile inspection after the plaster casts were removed on the sixth day after grafting. One week after all first-set skin allografts were rejected, during which time injections of thymosin, calf liver extract, BSA, or saline were continued, second-set skin allografts were applied to the same mice. Injections were continued until second-set allografts were rejected (plasters were removed on the fifth day after grafting). Total white cell and lymphocyte counts were done weekly on four mice from each group. White cell counts and lymphocyte counts were done daily

for 12 days in two nongrafted groups of ten $B_{10}D_2$/Sn mice each, one group receiving daily injections of 4.0 mg thymosin and the other receiving 4.0 mg BSA daily. Thymus, spleen, and lymph nodes from animals in each group were fixed in 10% formalin solution and sections stained with hematoxylin-eosin.

Allograft procedures; effects of antithymosin serum: The effect of ATS on survival of skin allografts was studied in 8–9-week-old male A/Jax mice. The animals were divided into three experimental and four control groups. One week prior to grafting, the experimental groups received daily subcutaneous injections of 0.15 ml of ATS while three control groups received 0.15 ml of NRS daily; an additional control group was not given injections. The A/Jax mice were then grafted with C57BL/6 skin. One group continued to receive daily injections of 0.15 ml ATS, a second group received 0.15 ml ATS every other day, while a third group was not given injections after receiving the graft. The three control groups received similar treatment with NRS on a parallel schedule and one group was not given injections. The care and evaluation of skin allografts were the same as that described previously for thymosin-injected mice. White cell counts and lymphocyte counts were performed on four mice in each group once a week. Daily blood samples were obtained from two nongrafted groups of 10 A/Jax mice each, one group receiving daily injections of 0.15 ml ATS and the other receiving 0.15 ml NRS daily. After second-set skin allografts were rejected, the animals were sacrificed and histological sections of thymus, spleen, and lymph node were prepared.

Results.—Thymosin-treated animals: Table 1 summarizes the mean survival times of skin allografts in $B_{10}D_2$/Sn mice receiving various doses of thymosin and compares these results with those obtained in control animals. In the C57BL/6 to $B_{10}D_2$Sn strain combination, the mean survival time for the first-set allografts was 10.2 ± 0.6 days, and for second-set grafts it was 7.3 ± 0.2 days. The rates at which first- and second-set allogeneic grafts were rejected in the animals injected with either the calf liver fraction, bovine serum albumin, or the two smaller doses (0.5 and 1.0 mg daily) of thymosin were the same as those in the mice injected with saline. In contrast, mice that received 4.0 mg of thymosin daily showed an accelerated rate of first- and second-set graft rejection. In these animals, the mean survival time of the allografts was 6.9 ± 0.9 days for first-set and 5.2 ± 0.2 days for second-set skin grafts. This represents a significant acceleration in skin allograft rejection in these thymosin-treated hosts for both first- and second-set skin allografts by 3.3 and 2.1 days, respectively. It may be noted that the mean survival time for second-set grafts in the control groups was almost the same as that for first-set grafts in the mice receiving 4.0 mg thymosin daily.

TABLE 1. *Effect of various dosages of thymosin on the rejection of C57/BL6 skin allografts by $B_{10}D_2$/Sn mice.*

Treatment*	Mean Survival Time†	
	First-set graft	Second-set graft
Saline (15)‡	10.2 ± 0.6	7.3 ± 0.2
Calf liver fraction, 2.0 mg daily (14)	10.4 ± 0.8	7.0 ± 0.8
Bovine serum albumin, 4.0 mg daily (12)	9.8 ± 1.1	7.1 ± 0.6
Thymosin, 0.5 mg daily (15)	10.5 ± 0.6	7.4 ± 0.9
Thymosin, 1.0 mg daily (16)	10.0 ± 1.0	7.4 ± 0.6
Thymosin, 4.0 mg daily (18)	6.9 ± 0.9	5.2 ± 0.2

* All animals were injected daily subcutaneously for 1 week prior to grafting and then daily until the rejection of second-set allografts. Each injection was in a volume of 0.2 ml 0.9% saline.

† Mean survival time in days ± standard error.

‡ Number in parentheses is number of animals in each group.

Figure 1 shows that $B_{10}D_2$/Sn mice which received either saline or calf liver extract rejected first-set allografts at the same rate. The same was true for mice receiving injections of BSA (not shown in Fig. 1). In contrast, mice that received 4.0 mg of thymosin daily showed an accelerated rate of first-set allograft rejection. Figure 2 presents data showing that mice that received 4.0 mg of thymosin daily also exhibited an accelerated rate of second-set skin graft rejection.

The total white blood cell and lymphocyte counts in the animals receiving thymosin did not differ significantly from the controls. The peripheral lymph nodes in the thymosin-treated animals were approximately three times larger than the nodes in the control animals. Preliminary histological examination under light microscopy revealed that the axillary and inguinal lymph nodes from thymosin-injected animals had larger germinal centers and were more densely populated with lymphoid cells in comparison to the nodes from control animals. The spleens in thymosin-injected animals also showed an increase in the number and size of germinal centers as compared to controls. The thymus from the experimental animals did not differ significantly from control thymus either in size or morphology.

Antithymosin-treated animals: Table 2 summarizes the mean survival times of first- and second-set skin allografts in the combination C57BL/6 and A/Jax strains of mice. In uninjected animals the MST for first-set grafts was 9.2 ± 0.6 days and for second-set grafts it was 6.8 ± 0.2 days. A/Jax mice which received normal rabbit serum rejected grafts at approximately the same time as mice given no serum. The mice treated with ATS only *prior to grafting* also rejected skin grafts at a time similar to the controls. A significantly prolonged survival of first- and second-set skin allografts was observed when ATS injections were *continued after grafting* and did not differ significantly whether ATS was given daily or every other day. The rate of allograft rejection in animals

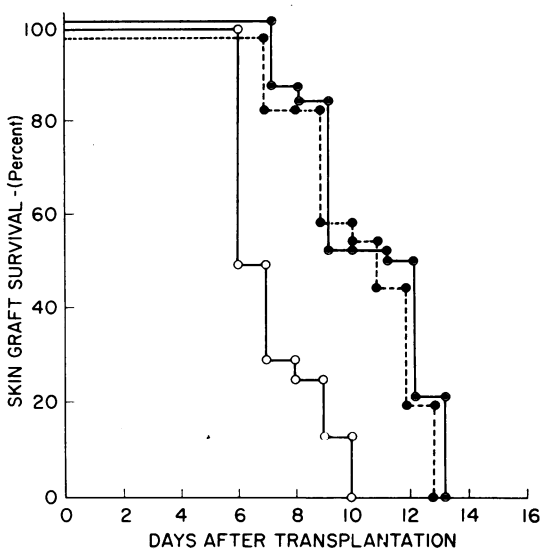

FIG. 1.—Effect of thymosin on first-set skin grafts. Adult $B_{10}D_2$/Sn mice were used as recipients of grafts, and C57BL/6 mice as donors. Prospective recipients were injected daily with 4.0 mg of thymosin (O—O), saline (●--●), or 4.0 mg of liver extract (◉—◉) beginning at 7 days prior to receiving the skin transplant, and injections were continued daily for the duration of the experiment. Beginning at 6 days after transplantation, each animal was examined and evaluations were made of the per cent of the skin area transplanted that had survived (ordinate). Each point is the average value for a group of 15 animals.

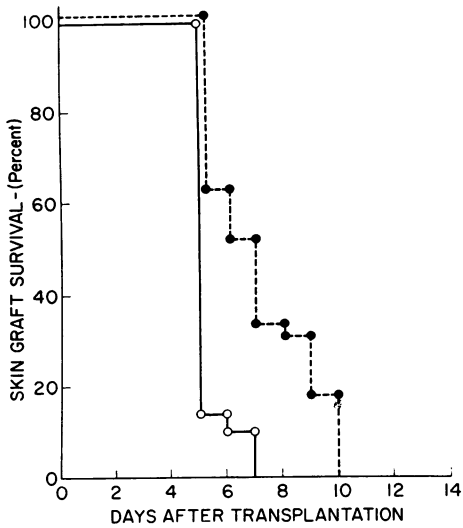

FIG. 2.—Effect of thymosin on second-set skin grafts; $B_{10}D_2$/Sn mice that had rejected a first-set skin graft from C57-BL/6 mice were utilized as recipients of a second C57BL/6 skin graft. Treatment, evaluation, and number of animals same as in Fig. 1, except that only one control group, which received saline injections (● - - -●), is presented; thymosin-treated animals (O—O).

receiving NRS and ATS is illustrated in Figure 3 for first-set grafts and in Figure 4 for second-set grafts (the results in noninjected animals are omitted in Fig. 4).

The daily white blood cell and lymphocyte counts of mice treated with ATS were not significantly different from those of mice treated with NRS.

Microscopic examination of thymic sections from mice treated with ATS revealed changes in the cortical and medullary regions when compared to sections from mice treated with NRS. The cortical area was crowded with thymic lymphocytes, whereas the medullary region was significantly reduced in size. Preliminary studies of sections of peripheral lymph nodes and spleen stained with hematoxylin and eosin revealed no significant differences between the animals injected with ATS as compared to controls.

Discussion.—The accelerated rejection of first- and second-set allografts of skin from C57BL/6 mice on $B_{10}D_2Sn$ mice after daily administration of large doses of thymosin as compared to rejection in animals receiving a fraction from liver prepared in a manner similar to thymosin, or BSA, suggests that thymosin has immunostimulatory properties. This observation is consistent with our previous

TABLE 2. *Effect of various schedules of treatment with antithymosin serum on the rejection of C57BL/6 skin allografts by A/Jax mice.**

Treatment after grafting	Mean Survival Time†			
	First-Set Graft		Second-Set Graft	
	NRS	ATS	NRS	ATS
None (12)‡	9.3 ± 0.9	9.4 ± 0.8	6.4 ± 0.4	6.7 ± 0.5
0.15 ml daily (15)	9.6 ± 0.4	16.4 ± 1.3	6.1 ± 1.4	9.2 ± 0.6
0.15 ml every other day (15)	9.8 ± 1.1	16.6 ± 1.6	6.6 ± 0.9	9.4 ± 0.9

* All animals (A/Jax) in this table were injected subcutaneously daily with either 0.15 ml normal rabbit serum (NRS) or with 0.15 ml antithymosin serum (ATS) for 1 week prior to grafting; subsequent treatment with NRS and ATS was as indicated. An additional group of 15 mice (not shown) was untreated either prior to or subsequent to grafting. The mean survival time in days of first- and second-set skin grafts in these animals was 9.2 ± 0.6 and 6.8 ± 0.2, respectively.

† Mean survival time in days ± standard error.

‡ Number in parentheses is number of animals in each group.

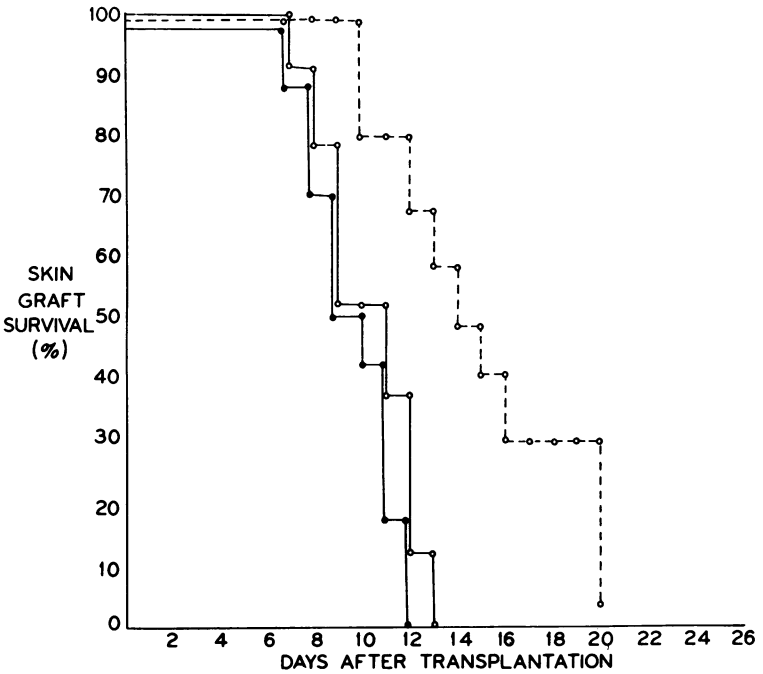

FIG. 3.—Effect of a rabbit antithymosin serum on first-set C57BL/6 skin grafts on A/Jax mice. Other details as in Fig. 1, except that groups of recipient mice either were not treated (O—O) or received either 0.15 ml normal rabbit serum (●—●) or 0.15 ml antithymosin serum (O- - -O) daily for 7 days prior to the allogeneic transplant and daily thereafter.

findings[1-6] that thymosin can influence various parameters of lymphoid structure and function.

The administration of 4.0 mg of thymosin daily to mice prior to and subsequent to allografting resulted in an accelerated rate of rejection of first-set and second-set allografts. These data might suggest that thymosin cross-reacts with the C57BL/6 skin transplantation antigen. However, smaller doses of thymosin did not accelerate allograft rejection. Nonetheless, further experiments are required to establish unequivocally that the accelerated allograft rejection produced by the large doses of thymosin is due primarily to its immunostimulatory action.

Thymosin administration increased incorporation of H^3-thymidine into peripheral lymph nodes[1-3] and also increased the weight and size of lymph nodes and spleens and the size of germinal centers in both these organs. It is suggested that large doses of thymosin may increase the number of immunocompetent cells in the lymphoid tissues, thereby making possible an earlier recognition of the allograft.

The observation that injection of an antiserum to a thymosin preparation (fraction 3) prolongs first- and second-set allograft survival suggests that the lymphoid cell involved in graft rejection can be selectively blocked by an antiserum prepared to a cell-free soluble thymic antigen.

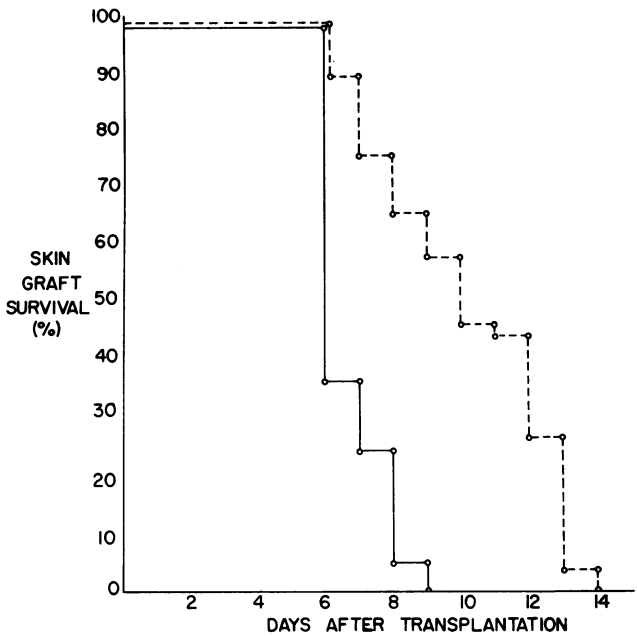

FIG. 4.—Effect of a rabbit antithymosin serum on second-set skin grafts. Other details as in Fig. 3, except that recipient A/Jax mice used had rejected a first-set skin graft and only two groups of animals are presented, one injected with 0.15 ml antithymosin serum daily (O- - -O) and the other injected with 0.15 ml normal rabbit serum daily (O—O).

Our results indicate that ATS must be given at frequent intervals to cause a prolongation of skin allograft survival and that ATS pretreatment alone is not sufficient to cause immunosuppression. The degree of prolongation of graft survival was not as striking as some descriptions of the effectiveness of antithymocyte serum[8, 12] or antilymphocyte serum.[13] This may perhaps be due to the small amount of ATS used in these experiments or to the fact that the ATS used was made against calf thymosin, or both. We have demonstrated[7] that an antiserum to calf thymosin is most effective in cytotoxic and agglutination reactions against calf thymocytes and lymphocytes. Although this antiserum also exhibited these reactions toward thymus cells of A/Jax mice, it was without effect against lymph node cells of species other than the calf. Thus this cytotoxic and leukoagglutinating capacity of ATS was not species-specific for thymocytes, supporting the concept of antigenic differences between lymph node cells and thymus cells.[14] It has been suggested[15] that thymocytes influence effector lymphoid cells either directly or through a humoral factor. It is therefore possible that the prolongation of skin allograft survival observed in the mice treated continuously with ATS was secondary to the antiserum's selective action on thymocytes. Alternatively, ATS may slow down the production of effector cells but may not alter or destroy those already present as part of the immune system of a competent animal. The action of the antiserum might be directed toward

inactivation of the small number of thymic cells actually involved in a delayed-type hypersensitivity reaction, or it might specifically inactivate the host's circulating titer of thymosin and hence render the animal less competent immunologically.

It should be emphasized that the thymosin preparation used in this study (fraction 3) contains several proteins as revealed by gel electrophoresis. Therefore, it is not known whether the ATS is directed against the same components responsible for the observed lymphocytopoietic and immunological effects of thymosin or toward other antigenic components of the fraction.

The histological changes observed in the thymus after treatment with ATS differ from those observed with antithymocyte serum. Turk and Willoughby[16] reported a depletion of lymphocytes in the thymic cortex of guinea pigs. Nagaya and Sieker[8] found a general depletion of small lymphocytes in the thymus gland of rats treated with antithymocyte sera. These findings contrast with the histological changes observed in this study in the thymus of ATS-treated mice; hypertrophy of the cortical region and a depletion of the medullary region were noted. It may be speculated that the apparent proliferation of cortical thymocytes is a feedback response due to inactivation of thymic dependent cells in the peripheral lymphoid system or possibly to a lowering of peripheral thymosin titer.

Studies by Mitchell and Miller[17] suggested that thymocytes, and to a lesser extent thoracic duct lymphocytes, are necessary for the maturation of bone marrow cells to antibody-producing cells. In view of their evidence and our own results, we suggest that thymosin, a partially purified soluble extract of thymus, may be causative in the production of "recognition" cells to an antigenic stimulus. Thus thymosin may be an important factor in the early formation of multipotential "recognition" cells which probably are required in an initial step in graft rejection.

Summary.—Administration of a soluble lymphocytopoietic fraction of calf thymic tissue designated as thymosin to host mice accelerated rejection of first- and second-set skin allografts. In contrast, injection of a rabbit antiserum prepared against a calf thymosin fraction significantly prolonged the survival of first- and second-set skin allografts. These observations, coupled with histological examination of thymic and lymph node tissue of injected host animals, suggest that the action of thymosin and its antiserum includes an influence on lymphoid cells concerned with expression of host-specific immune phenomena.

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