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Failure of Thymopoietin, Ubiquitin and Synthetic Serum Thymic Factor to Restore Immunocompetence in T-Cell Deficient Mice (40313)

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The role of the thymus in lymphocyte homeostasis (1, 2) and in conversion of precursor lymphocytes to thymus-dependent lymphocytes (T cells) has been the subject of intensive investigation during the last 20 years. Absence of the thymus leads to diverse immunologic deficiencies that can be reversed by thymus grafts or by thymus grafts enclosed in cell-impermeable chambers (3–7), suggesting that the thymus might induce maturation of T lymphocytes through production of a soluble factor(s). Candidate thymic factors have been prepared by several investigators. The biological activities of these factors have been assessed mainly by *in vitro* induction of T cell markers (e.g. thy-1 antigen) on lymphocyte populations (8–12), with little or no attention given to whether there was restoration of thymus-dependent immune functions measurable *in vivo*.

The present study was undertaken to evaluate several substances that induce T cell markers for their ability to restore thymus-dependent immunocompetence in thymectomized C58 mice. The substances tested were thymopoietin and ubiquitin, prepared by Dr. G. Goldstein (10, 11), and synthetic serum thymic factor, defined by Bach *et al.* (13).

Materials and methods. Mice. C58/J mice were obtained from the closed colony maintained for the Merck Sharp & Dohme Research Laboratories by Buckshire Farms, Perkasie, PA or were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Material evaluated. Partially purified thymopoietin II (10) and ubiquitin (11) were provided as aliquoted lyophilized preparations by Dr. Gideon Goldstein, Sloan-Kettering Cancer Research Center, New York. Samples to be tested were dissolved in phosphate buffered saline (PBS) immediately prior to use. Mice were injected intraperitoneally with the substances in 0.1 ml. Synthetic serum thymic factor (Pyroglu-Ala-Lys-Ser-Glu-Gly-Gly-Ser-Asn), defined by J-F Bach *et al.* (13), was synthesized by the peptide synthesis group (R. G. Strachan, W. J. Paleveda, S. J. Bergstrand, R. F. Nutt, R. Hirschmann, F. W. Holly, and D. F. Veber; manuscript in preparation), of Merck Sharp and Dohme Research Laboratories, and found positive for *in vitro* biological activity by Dr. J-F Bach, Necker Hôpital, Paris, France. Dosages, formulations and frequencies of treatment employed in these experiments were based on recommendations made by Dr. G. Goldstein for thymopoietin and ubiquitin, and by Dr. J-F Bach for serum thymic factor.

An unrelated pentapeptide, Asp-Ser-Asp-Pro-Arg (14), and a decapeptide, Val-His-Leu-Ser-Ala-Glu-Glu-Lys-Glu-Ala (15), that failed to show *in vitro* biological activity in tests performed by Dr. J-F Bach were used for control purpose.

Thymectomy. Mice that were 0–2 days old were anesthetized by cooling at −20° (16) for 5–8 min (depending on size), thymectomized according to the method of Sjodin *et al.* (17), and then warmed under an infrared lamp (35°C) for 30 minutes. Young adult (4- to 6-week-old) mice were anesthetized by a single intraperitoneal injection (62.5 mg/kg) of sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, Illinois), and thymectomized according to the method of Dardenne and Bach (18). Sham thymectomized mice were treated surgically in the same manner except that the thymic lobes were not removed. At the appropriate time, all thymectomized mice were examined for presence of thymic remnants with the aid of a dissecting microscope. Mice found to have thymic remnants were excluded from the study.

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Anti-thymocyte serum treatment. Heat-inactivated rabbit anti-mouse thymocyte and normal rabbit sera were purchased from Microbiological Associates, Bethesda, Maryland. A single 1 ml injection of serum was given intraperitoneally 3–4 days after thymectomy. Certain lots of anti-thymocyte sera were toxic for the mice and were not used.

Preparation and administration of I_b cell suspensions. The challenge inoculum was made by mixing equal volumes of viable (2×10^3/ml) and irradiated (2×10^6/ml) line I_b leukemic cell suspensions. Suspensions of viable (C58 mouse-derived) I_b cells were prepared in Hanks balanced salt solution as described previously (19). To prepare γ-irradiated I_b cells, suspensions of viable cells were exposed to 10,000R in a Model 109 Co^{60} Irradiator (J. L. Shepherd and Assoc., Glendale, CA) that delivered 62,000R/min. Mice were injected intraperitoneally with 1 ml of the I_b cell mixture (10^3 viable plus 10^6 irradiated cells). The mice were observed for 21 days and gross examination of the viscera of all mice that died was made to assure that deaths were due to leukemia.

Mitogenic responses. To test for capability to respond to mitogens, spleen and mesenteric lymph node cell suspensions were prepared in medium RPMI 1640 (Grand Island Biological Co., Grand Island, NY) containing 5% fetal calf serum (Microbiological Associates). Five replicate cell suspensions, each containing 4×10^5 cells in 0.2 ml, were prepared for testing the response to concanavalin A (Con A) (Miles Laboratories, Kankakee, IL) and phytohemagglutinin P (PHA) (Difco, Detroit, MI) in final concentration of 0.4 μg/ml and 1:1000 dilution, respectively. After 3 days of incubation (37°, 5% CO_2), 1 μCi of tritiated thymidine (New England Nuclear, Boston, MA) in 0.025 ml was added to each cell preparation, and incubation was continued for an additional 4 hr. The cells were harvested, washed to remove residual free fluids, and dissolved in 10 ml of Scintisol (Isolab, Akron, OH). The counts per minute were determined, and the mean cpm was calculated for the 5 replicate cultures in each group.

Results. Failure of thymopoietin to restore T cell mitogen responses of lymphocytes from neonatally thymectomized C58 mice. Findings in preliminary experiments indicated that both spleen and lymph node cells from untreated or PBS-treated neonatally thymectomized C58 mice failed to be stimulated by T cell mitogens. In fact, incubation with Con A or PHA generally resulted in decreased thymidine incorporation compared to that of control cells that were not treated with mitogen.

To test for ability of thymopoietin to restore T cell mitogen responses, neonatally thymectomized C58 mice were treated daily with thymopoietin for 4 weeks starting at 1 week of age. The animals were sacrificed by cervical dislocation 1 day following the last injection. Spleen and lymph node cells were removed from the animals and tested for mitogenic responses to Con A and PHA. As shown in Table I, treatment with thymopoietin did not restore normal responsiveness of the spleen and lymph node cells to the T cell mitogens.

Failure of thymopoietin and ubiquitin to restore resistance to line I_b leukemia in adult thymectomized C58 mice. It was demonstrated in previous studies that normal adult C58 mice develop an immune response (survival) when simultaneously vaccinated and challenged with a mixture of viable and killed line I_b leukemic cells, whereas immunosuppressed mice do not (19, 20). This immune response is highly dependent on functional maturity of the T-lymphocytes (21). An experiment was carried out in which adult thymectomized and sham thymectomized control animals were treated with rabbit anti-thymocyte serum to reduce the population of competent lymphocytes in the periphery. The animals were then challenged with the I_b cell preparation described above. As shown in Fig. 1, sham thymectomized animals were initially highly susceptible to challenge with I_b cells but their immunologic responsiveness was regained within 4 weeks after serum administration. By contrast, animals that had been thymectomized did not regain their resistance. Similar differences in regeneration of T cell mitogen responses (22) and thy-1-bearing lymphocytes (23) were observed between adult thymectomized and sham thymectomized mice given anti-thymocyte serum.

An attempt was made to restore the im-

TABLE I. FAILURE OF THYMOPOIETIN TO RESTORE T CELL MITOGEN RESPONSIVENESS IN NEONATALLY THYMECTOMIZED C58 MICE.

In Vivo treatments[a]		In Vitro mitogen responses[b]					
		Spleen Cells			Lymph Node Cells		
Thymectomy	Substance	Control cpm (No. mice)	PHA stimulation index	Con A stimulation index	Control cpm (No. mice)	PHA stimulation index	Con A stimulation index
unoperated	None	3322 (5)	3.3	32.7	63 (6)	30	183
NTx	TP	4451 (8)	0.6	1.1	2878 (6)	0.1	0.5
NTx	PBS	4383 (2)	0.4	0.5	132 (2)	4.3	ND
NTx	None	4610 (8)	0.8	1.7	2677 (6)	0.7	0.4*

[a] Neonatally thymectomized (NTx) C58 mice were treated ip with 1 μg thymopoietin (TP) or with PBS 5×/week for 4 weeks (20 treatments) starting at 1 week of age.

[b] Averages of individual determination obtained from the indicated numbers of mice. Stimulation Index = Mitogen Stimulated cpm/control culture cpm

* Data from two animals.

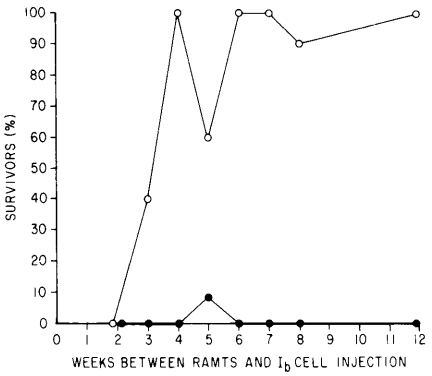

FIG. 1. Effect of adult thymectomy on recovery of the immune response to line I_b leukemic cells following anti-thymocyte serum treatment. Groups of thymectomized (●) and sham thymectomized (○) mice were injected with an admixture of 10^3 viable I_b cells and 10^6 γ-irradiated I_b cells at the indicated times after the injection of 1 ml rabbit anti-mouse thymocyte serum. Each point represents 20 mice. Groups of thymectomized mice given normal rabbit serum survived the injection of I_b cells.

munologic responsiveness of anti-thymocyte serum-treated adult thymectomized C58 mice by administration of thymopoietin or ubiquitin. Such mice were injected intraperitoneally with 1 μg thymopoietin or ubiquitin 5 times per week for 5 weeks prior to challenge with I_b cells. Neither thymopoietin nor ubiquitin restored the resistance of serum-treated thymectomized animals to line I_b leukemia (Table II). On the other hand, control serum-treated sham thymectomized mice were resistant to challenge.

Failure of serum thymic factor to restore resistance to line I_b leukemia in adult thymectomized C58 mice. In similar experiments to those described above, anti-thymocyte serum-treated adult thymectomized C58 mice were injected 3 times per week for 8 weeks with synthetic serum thymic factor (0.1 ng) prepared with carboxymethylcellulose as described by M-A Bach (24). T cell immunocompetence was measured in terms of the survival rates of animals challenged with line I_b leukemia. As shown in Table III, resistance to challenge was not restored to serum-treated thymectomized mice by treatment with serum thymic factor. Control animals that had been sham thymectomized and treated with anti-thymocyte serum were resistant to challenge.

In the experiments just described, repeated injections of carboxymethylcellulose, in which the test preparations were suspended, was toxic; causing skin nodules, ulceration and death in roughly half the animals during the 8 week period of treatment. To avoid this, adult thymectomized animals that had been given anti-thymocyte serum were treated 5 times per week for 8 weeks with 1 μg serum thymic factor in PBS and then challenged

TABLE II. LACK OF EFFECT OF THYMOPOIETIN AND UBIQUITIN ON RESISTANCE OF ANTI-THYMOCYTE SERUM-TREATED ADULT THYMECTOMIZED MICE TO CHALLENGE WITH LINE I_b LEUKEMIA.

Treatment of mice		Response to line I_b leukemia[c]	
Pre-Therapy T Cell Depletion[a]	Substance Tested[b]	No. of surviving/total (%)	Average time of death in days (± SD)
	Thymectomized test animals		
ATx, RAMTS	Thymopoietin	0/16 (0)	10.69 ± 0.60
ATx, RAMTS	Ubiquitin	1/13 (8)	10.69 ± 0.78
	Thymectomized control animals		
ATx, RAMTS	PBS	0/12 (0)	10.75 ± 1.06
ATx, RAMTS	None	0/13 (0)	10.92 ± 1.75
	Nonthymectomized control animals		
STx, RAMTS	PBS	11/12 (92)	*
Unoperated	PBS	20/20 (100)	

[a] C58 mice were adult thymectomized (ATx) or sham operated (STx) and given rabbit anti-mouse thymocyte serum (RAMTS) 3 days later.

[b] Treated mice received 1 μg of thymopoietin or ubiquitin ip 5×/week for 5 weeks.

[c] All mice were challenged with a mixture of 10^3 viable and 10^6 irradiated I_b cells.

* One mouse died on day 15.

TABLE III. LACK OF EFFECT OF COMPLEXED SERUM THYMIC FACTOR ON THE RESISTANCE OF ANTI-THYMOCYTE SERUM-TREATED ADULT THYMECTOMIZED MICE TO CHALLENGE WITH LINE I_b LEUKEMIA.

Treatment of mice		Deaths following treatment with the test substances in CMC			
		No. of mice			Survival following challenge with line I_b leukemia (%)
Pre-therapy T cell depletion[a]	Substance tested[b]	Start	Final	Survival (%)	
	Thymectomized test animals				
ATx, RAMTS	Serum thymic factor	15	7	(46)	1/7 (14)
ATx, RAMTS	Decapeptide	15	12	(80)	4/11 (36)
	Thymectomized control animals				
Atx, RAMTS	Buffered saline solution	15	8	(53)	0/7 (0)
	Sham thymectomized control animals				
STx, RAMTS	Buffered saline solution	15	9	(60)	9/9 (100)

[a] C58 mice were adult thymectomized (ATx) or sham operated (STx) and given rabbit anti-mouse thymocyte serum (RAMTS) four days later.

[b] All test substances were contained in carboxymethylcellulose (CMC) that was highly toxic, causing deaths in the animals. Treated mice received 0.5 ml sc containing 0.1 ng of serum thymic factor or control decapeptide combined with 27 mg CMC. Treatment was started 6 days following RAMTS treatment and continued three times per week for a total of 20 injections. CMC and total volume of treatment were reduced to 5 mg and 0.1 ml, respectively, after seven injections.

with I_b cells. The findings given in Table IV show that the thymic factor in PBS, as in carboxymethylcellulose, failed to restore immunocompetence to the mice.

Discussion. The main criterion used to classify materials as thymic hormones has been their capacity to induce T cell surface membrane markers on lymphocytes. However, the induction of such cell markers seems not to reflect a maturation event specifically induced by thymic hormone, since many unrelated substances, including nonthymic tissue extracts, ubiquitin, poly A:U, endotoxin, prolactin, glucagon, prostaglandin E and histamine, all have the ability to induce the same cell surface markers (11, 25, 26). Therefore, it is of value to test candidate thymic hormones in more discriminating assays; assays that would measure the effect on immunocompetence. The present studies were carried out to measure the ability, if any, of thymopoietin, ubiquitin, and serum thymic factor to restore immunocompetence *in vivo* in T lymphocyte deficient C58 mice. Daily admin-

TABLE IV. LACK OF EFFECT OF UNCOMPLEXED SERUM THYMIC FACTOR ON THE RESISTANCE OF ANTI-THYMOCYTE SERUM-TREATED ADULT THYMECTOMIZED MICE TO CHALLENGE WITH LINE I_b LEUKEMIA.

Treatment of Mice[a]		Deaths following treatment with the test substances			
		No. of mice			Survival following challenge with line I_b leukemia (%)
Pre-therapy T cell depletion	Substance tested	Start	Final	Survival (%)	
Thymectomized test animals					
ATx, RAMTS	Serum thymic factor	13	9	(70)	2/9 (22)
ATx, RAMTS	Pentapeptide	13	12	(92)	3/12 (25)
Thymectomized control animals					
ATx, RAMTS	Buffered saline solution	13	13	(100)	4/13 (31)
Sham thymectomized control animals					
STx, RAMTS	Buffered saline solution	13	12	(92)	12/12 (100)

[a] Mice were treated as indicated in Table III except that each mouse received 0.1 ml PBS sc containing 1 ng of serum thymic factor or control pentapeptide five times per week for a total of 36 injections.

istration of thymopoietin to neonatally thymectomized C58 mice for 4 weeks did not restore the responsiveness of their lymph node and spleen cells to T cell mitogens. Further, daily treatments of anti-thymocyte serum-treated adult thymectomized C58 mice with thymopoietin, ubiquitin, or serum thymic factor were ineffective in restoring the capacity of these mice to resist challenge with line I_b leukemia. By contrast, sham thymectomized C58 mice, that had received anti-thymocyte serum, recovered their resistance to line I_b cell challenge spontaneously within 4 to 5 weeks after serum treatment. Thus, thymopoietin and serum thymic factor, in the regimen used, did not mimic the restorative function provided by the intact thymus.

The failure of the candidate thymic factors under investigation to restore immunocompetence in T cell deficient mice might be due either to insufficient exposure of the precursor cells to these substances or to total irrelevance of the substances to the cell maturation process. The more likely explanation, however, as suggested by A. L. Goldstein *et al.* (27), is that immunologic maturation is a process involving a number of steps and that a single factor, initiating a single cellular event, might not be reflected in any meaningful immunologic activity. Scheid *et al.*, for example, have demonstrated that *in vivo* (28) or *in vitro* (26) treatment with thymopoietin induces TL and thy-1 antigens on murine lymphocytes. However, lymphocytes that carry the TL marker are known to be immature and immunoincompetent (29). Similarly, Bach *et al.* (8, 24) have shown that serum thymic factor can maintain a normal level of short-lived thy-1-positive lymphocytes in adult thymectomized mice. Yet, the responses of adult C58 mice to T cell mitogens and line I_b leukemia are dependent on a long-lived population of thy-1-positive lymphocytes. Perhaps the evaluation of only one substance at a time would inevitably result in failure to induce immunocompetence.

Abstract. Thymopoietin, ubiquitin, and serum thymic factor, all of which induce T cell markers on lymphocytes, have been evaluated for their capacity to induce thymus-dependent activities *in vivo*. Multiple treatments over a period of weeks failed to restore either resistance to line I_b leukemia or responses to T cell mitogens in T cell-deficient C58 mice. The findings suggest that these substances are ineffective in inducing thymus-dependent immunocompetence that is meaningful in the intact animal.

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