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Response of Spleen Lymphocytes Shortly After Thymectomy in Adult Mouse

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The blastogenesis response to the phytomitogens, PHA-P, Con A and PWM was used to assess the effect of adult thymectomy on the spleen lymphocytes of C57B1 mice.

The mitogenic response to the phytomitogens was determined by ^{3}H-thymidine uptake. The changes produced in theta-antigen bearing spleen lymphocytes were also evaluated making use of theta antibodies from AKR/S mice previously injected with splenic and thymic lymphocytes from CBA/J mice.

The present results show that the response to mitogens PHA-P and Con A is reduced early after thymectomy while the response to PWM was only slightly reduced. There was not any correlation between tthe disminished response to mitogens and the changes observed in theta bearing spleen lymphocytes.

Older studies on adult thymectomized animals have demonstrated that there is a slow decline in humoral and cell-mediated immune responses to particulate and soluble antigens (8, 9, 18). More recent studies have demonstrated that there may also be some striking effects early after adult thymectomy: *a*) a 50 % loss of theta bearing spleen lymphocytes 27 and 55 days after thymectomy (12). *b*) Simultaneous inability to develop immunologic memory to sheep erythrocyte antigens (11), and *c*) spontaneous rosette forming cells, which bear the theta antigen, and which are sensitive to inhibition by low concentrations of azathioprine or anti-lymphocyte serum disappeared within 5 days after adult thymectomy (2). Finally, the *in vitro* analogue of cell-mediated immunity testing, the mixed lymphocyte reaction, was significantly supressed as early as four weeks after thymectomy of adult rats (14). The present report deals with the early loss in the mitogenic response of spleen lymphocytes to phytohemagglutin-P (PHA-P and Concanavalin A (Con A) shortly after adult thymectomy.

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and confirms the above observations suggesting that there is an early loss of peripheral T cells.

Materials and Methods

C57B1/6J (2-5 months old) male mice from Jackson Laboratories were used throughout this study. They were fed with Purina chow and water *ad libitum*. In a give experiment the mice were of the same age.

Thymectomy. Mice were given sodium nembutal (Abbott, 60 mg/kg body wt.). The method used for thymectomy was a suction procedure previously described by SJORIN *et al.* (15). It was found that by slowly pushing the forceps in a depth of about one quarter inch with repeated expansion the thymus could be more easily removed by suction. The thymus was removed by placing a glass tube (the tip of which was drawn to a diameter of about 1/8 of an inch) under the sternum to a depth of one quarter inch. Suction was applied by a vacuum pump Type KS, 1/10th Horsepower (Robbins and Myers, Sprindfield, Ohio) at a pressure of 15 psi. After the thymus entered the tip of the tube, slow withdrawal with upward pressure generally resulted in complete removal of the thymus. It should be noted that this is a blind operation. Animals that showed residual thymus tissue upon gross examination were not used in experiments. In sham thymeoctomized animals the identical procedure was followed, including traction on the gland. 5 to 8 weeks old mice were used.

Tissue culture medium. RPMI-1640 powered medium (Grand Island reagents were used throughout the study except where otherwise stated) was reconstituted with approximately 300 ml of distilled water to which was added 26.3 ml of 7.5 % sodium bicarbonate followed by adjustment of the pH to 7 with 1N HCl. Ten ml of 1 Molar HEPES (Microbiological Associates) was then added followed by distilled water to 500 ml. The 2X medium was sterilized by filtration through 0.45 micron Millipore filters (Falcon filter n.° 7102) and was stored at 4° C until the moment of the experiment.

When the above described tissue culture medium was used 50 ml of 2X medium was removed to which was added 1.0 ml of penicillin-streptomycin solution, 5.0 ml of heat inactivated (56° C for 30 minutes) fetal calf serum (Rehatuin FS, Reheis Chemical Co., Chicago) and distilled water to complete 100 ml, followed by sterilization as above described.

Preparation of mitogens. Phytohemagglutinin-P (PHA-P, Difco Labs., Detroit) was dissolved by adding 5.0 ml of sterile distilled water (Grand Island) to the vial. This was diluted to 1:5 with distilled water and was sterilized by filtration, distributed in small aliquots, and frozen at —85° C. When this was employed further dilutions were made with sterile distilled water and 0.01 ml of each final solution was added to the lymphocyte cultures. The final dilution obtained represents the total dilution of PHA-P prior its addition to cultures.

Concanavalin A (Con A), 3X crystallized was obtained from Miles Laboratories, Inc. (Kankakee, Ill.). It was dissolved in Dulbecco's phosphate buffered saline (DPBS, Grand Island), and the protein concentration was determined at 280 mμ using 1.3 optical density units per mg of protein. A stock solution of 100 mμ/ml was prepared from this value, sterilized by filtration and stored in small portions at —85° C. At the moment of use the stock solution was diluted to various concentrations with sterile DPBS. A volume of 0.1 ml was then added to the lymphocyte cultures with control receiving 0.1 ml of diluent.

Pokeweed mitogen (PWM, Grand Island) was dissolved with 5.0 ml sterile

distilled water, alliquoted in small portions and stored at —20° C. When used, various dilutions were prepared with tissue culture medium, and 0.1 ml was added to each culture with controls receiving medium alone.

Stock mitogen solutions were discarded after the first thawing and never were re-used.

Lymphocyte culture for induction of mitogenic response. The lymphocyte culture method is a modification of that of Janossy and Greaves (7). Spleens were removed from mice by sterile technique, and placed in sterile petri dishes containing 5 ml of RPMI-1640. The spleens were pressed between the blades of large forceps (Eastern Scientific, Providence, R.I.). After removing the connective tissue, the cells were brought into sterile vacuum tubes (vacutainer n.° 40, yellow stopper) using 20G multi sample vacutainer needle (Becton, Dickinson and Co.). With a 5.0 ml disposable syringe and a 20G needle the suspension was aspirated 7-8 times in order to prepare a single cell suspension. The suspension was then transferred to a sterile glass screw top centrifuge tube and was sedimented at 500 rpm for 10 minutes in a PR-6 International Centrifuge (head n.° 269). The supernate was discarded and the pellet was suspended with 1.0 ml of One ml of this suspension was placed in preparing the suspension. The number of viable cells/ml was determined by trypan blue viability, and the suspension was adjusted to contain 2.5×10^6 cells per ml. One ml of this suspension was placed in wells of plastic disposable tissue culture plates (Linbro Plastics Model FB-16-24-TC, maximum well volume of 3.5 ml). Mitogen (or diluent for controls) was then added in a volume of 0.1 ml (or 0.01 ml) in various experiments. The plates were rotated to mix cells and mitogen and were incubated at 37° C for 2 or 3 days in a humidified atmosphere of 95 % air, 5 % CO_2 or 83 % N_2, 10 % CO_2, 7 % O_2.

Preparation of theta antibody (AKR anti-theta C_3H). Thymus cells from CBA/J male mice were separated as were done with spleen cells in Hank's BSS. After trypan blue viability count the suspension was adjusted to 2×10^7 cells per ml. One ml of freshly made suspension was injected into AKR/J male mice (with ages ranging from 6 to 10 weeks) each week for 6 consecutive weeks (12). Ten days after the last injection the mice were bled by cardiac puncture under ether anesthesia. The serum was frozen in small aliquots until the moment of use.

Determination of theta-bearing spleen cells. Since C57Bl/6J mice were used in the present study, spleen cells from these mice were used to determine the potency of the serum. In 12 × 75 mm test tubes 2×10^6 spleen lymphocytes in 0.1 ml were placed. 0.1 ml of the test serum or normal AKR serum (for controls) was added to each of two tubes containing spleen cells that were incubated at 37° C for 30 minutes. After centrifugation at 500 rpm for 10 minutes the supernate was removed. It was added 0.3 ml of 1:4 dilution of guinea pig complement adsorbed with agar (3). The tubes were moved and incubated for a further 30 minutes at 37° C after which they were chilled in a 4° C water bath. Percent kill was then assessed by trypan blue uptake. Subtraction of background kill due to normal AKR serum from percent kill with AKR anti-theta C_3H showed that this serum killed 25-35 % of normal C57 spleen cells. This procedure was used in assessing changes in theta bearing spleen lymphocytes in thymectomized mice.

Assessment of mitogenic response. The response to all mitogens was determined by uptake of tritiated thymidine into DNA (4). Cultures were assayed on day 2 or 3 after initiation. One μC of tritiated thymidine (New England Nuclear, sp. act. 20C/mM) in 0.1 ml was added to each culture

and the mixture was incubate for a further 4 hours. DNA was extracted on Millipore filters previously soaked in a solution of unlabeled thymidine (2 mg/ml). Prior to extraction each culture received 1 ml of unlabeled thymidine (2 mg/ml) and DNA was extracted by washing each culture with saline throught 25 mm 0.45 micron filters held on a Millipore suction apparatus. The cells were then washed twice with 10 ml of normal saline, twice with 2.0 ml of cold 5 % trichloracetic acid and twice with 2.0 ml ethanol (95 %). The filters were placed in plastic scintillation vials to which was added 10 ml of Aquasol (New England Nuclear). The samples were counted on a Beckman LS230 liquid scintillation counter.

Results

A number of control experiments were initially done in order to obtain optimum conditions in our laboratory. This was deemed necessary since one recent investigation (1) reported that fresh heat inactivated human serum was needed for optimum blastogenesis in a gas environment of 5 % CO_2 in air. Another report (7) showed that fetal calf serum could be used successfully under an environment of 7 % CO_2, 10 % O_2, and 83 % N_2, a gas shown to be important for induction of *in vitro* immune responses by mouse lymphocytes (10). Our studies (unpublished) demonstrated that fetal calf serum (Rehatuin, F.S.) is as good as human serum in the culture system. In the both groups above (1, 7) were used cell concentrations of approximately 5×10^6 cells/ml of culture. Tables I and II depict typical experiments showing that 2.5×10^6 cells/ml were better than 5×10^6, as well as the fact the gas environment did not appear to effect blastogenesis. Though these experiments were done with PHA-P, a concentration of 2.5×10^6 cells/ml was used for most experiments with PWM and Con A as well as a gas environment of 5 % CO_2 in air. Specific alterations are noted in captions and legends.

In this study, the total of 34 spleen were used. In each of them the spleen lympho-

Table I. *Effect of PHA-P concentration and cell density on reactivity of normal spleen cells* Assayed on day 2. Spleen cells from one mouse were used.

N.° of cells/ culture	Dilution of PHA-P *				
	1/30	1/40	1/60	1/80	0
			(cpm ± S.D.)		
5×10^6	50521.2± ±9141.8	66318.3± ±23768.6	69993.3± ±4958.1	65179.1± ±5858.1	7101.0± ±1378.5
2.5×10^5	87706.2± ±23613.9	101141.6± ±26943.7	117600.0± ±5053.9	136287.5± ±23433.9	7172.9± ±1349.7

* 0.01 ml of each dilution was added to each of three replicate cultures.

Table II. *Effect of gas on blastogenesis.*

Gas * %	Dilution of PHA-P **	
	1/80	0
	(cpm ± S.D.)	
83 N_2 + 10 CO_2 + 7 O_2	23640.7±1142.0	4577.2±544.0
95 Air + 5 CO_2	25354.4±3936.4	3802.6±638.5

* Triple gas mixture obtained from Matheson Gas Products (East Rutherford, N.J.), 5 % CO_2 in Air was obtained from Linde, Div. of Union Carbide Corp. (New York, N.Y.).
** 0.01 ml of PHA-P added to each culture.

cytes were cultivated with the 3 mitogens by triplicate.

It was observed that with any given dose of mitogen the extent of incorporation of tritiated thymidine (^{3}HT) was quite variable between experiments conducted on different days and thus with different spleen lymphocyte populations. The data in figure 1 (and also table I for PHA-P) shows the effect of mitogen dose on the response of spleen cells when were assayed on day 2 and 3. With PHA-P and PWM (bottom, fig. 1) there was a relatively wide optimum dose range, whereas with Con A (top, fig. 1) there seemed to be a narrow range. This observation was quite consistent in many experiments, though in some, narrower ranges than reported here were seen for PHA-P and PWM. The optimum dose chosen for PHA-P, Con A and PWM was rather arbitrary in these studies. In general, the third day assays showed lower responses, even though this is less apparent with PHA-P data shown here.

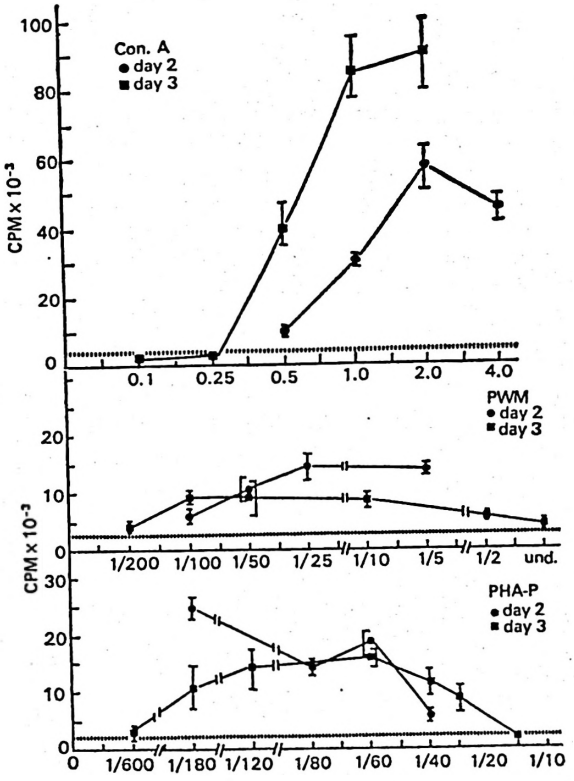

Fig. 1. *Reactivity of normal spleen cells to varying doses of mitogens.*
Con A (top), PWM (middle) and PHA-P (bottom). Separate spleen cell populations were used for day 2 and day 3 assays. The dose (abscissa) for Con A is in μg, whereas for PWM and PHA-P are in dilutions (see methods). The response of control cultures without added mitogens is represented by the broken line and includes the mean of day 2 and day 3 cultures (3 replicates each day) since the ranges both days were similar.

The response to the three phytomitogens at various times after adult thymectomy as well as changes in splenic theta antigen bearing cells are shown in figure 2 and table III. At each time interval after thymectomy the same spleen cell population was tested with all of the three mitogens; and was assayed on either day 2 or day 3. The exception is at 11 weeks post-THX. Here the same cells were assayed on days 2 and 3 (fig. 2) and the results obtained with three different doses of each mitogen with these cells are summarized in figure 3.

It is clear that by three weeks after thymectomy splenic lymphocytes did not respond well to PHA and Con A, whereas with two exceptions, at 8 and 14 weeks, the response to PWM was only slightly supressed. It is difficult to explain why a 1 and 14 weeks the response to Con A

Table III. *Effect of thymectomy on theta-bearing spleen cells.*

Weeks after thymectomy	Theta-bearing cells * (per cent)	
	Normal mouse	Thymectomized mouse
3	28	13
8	19	22
10	25	17
11	16	14
12	28	12

* Per cent determined after subtraction due to kill with normal AKR serum.

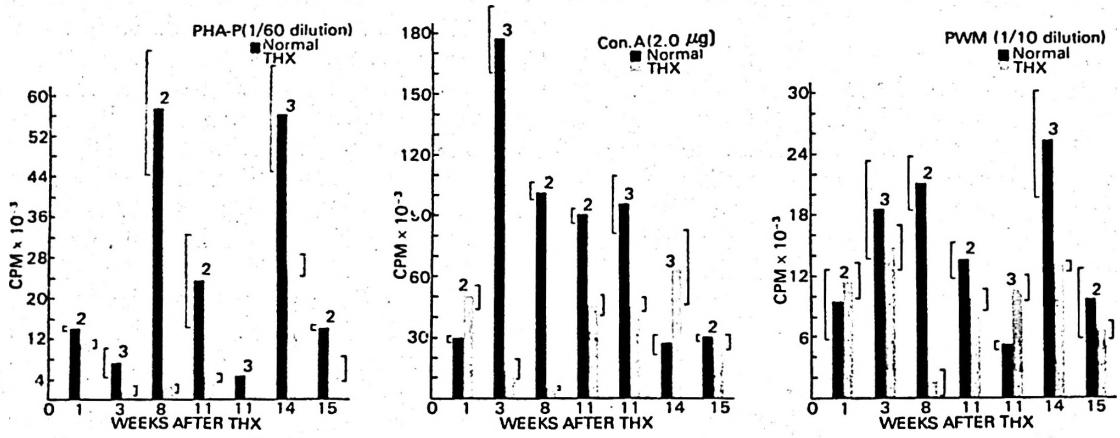

Fig. 2. *The response to PHA-P (left), Con A (middle) and PWM (right) at various times after adult thymectomy.*

The numbers at the top of each bar refer the day of assay. The vertical bars are standard deviations of three replicate cultures. Note that at 11 weeks post-THX the assays were done on both days 2 and 3. For reasons of clarity the response of cultures without mitogens are not included. The range of the means of three replicate cultures of normal cells was from 332.6 to 3734.8 and for THX spleen cells from 396.6 to 3158.9

by THX spleen cells is greater than in normal mice. This could reflect technical problems which should be clarified by continuing experiments. The supressed response to PHA-P was the most consistent.

The variations in the dose of each mitogen did not seem to change the response of spleen cells 11 weeks post-THX (fig. 3), whereas for normal spleen cells there were sharper changes. This figure also shows the low response of the cells without added mitogen, and even here the response of normal spleen cells was slightly greater than THX spleen cells response.

The results of analysis of theta-bearing cell content (table III) did not correlate with the mitogenic responses. A notable decline was seen at 3, 10 and 12 weeks, but at other times there was not a difference.

It should be noted that thymectomized mice were assayed at 3, 8 and 11 weeks post-THX for response to the mitogens (fig. 2) and only the mice assayed at 3 weeks post-THX showed a decline in the number of theta-bearing cells.

In table IV are compared the results of the response to the three mitogens in a thymectomized mouse and in a sham-

Table IV. *Response to mitogens 4 weeks after thymectomy.*

The concentrations of mitogens were: PHA-P, 0.01 ml of a 1/60 dilution; Con A, 2 μg in 0.1 ml; PWM, 0.1 ml of a 1/10 dilution. See methods section for details. Thymectomy and sham was done on 6 weeks old mice.

	Mitogens			
Mouse	PHA-P	Con A	PWM	0
Thymectomized	686.0±310.3	9146.5±2276.4	609.6±197.7	584.0±127.4
Sham	2567.3±820.8	22788.0±3916.0	1455.0±384.0	745.6±621.4

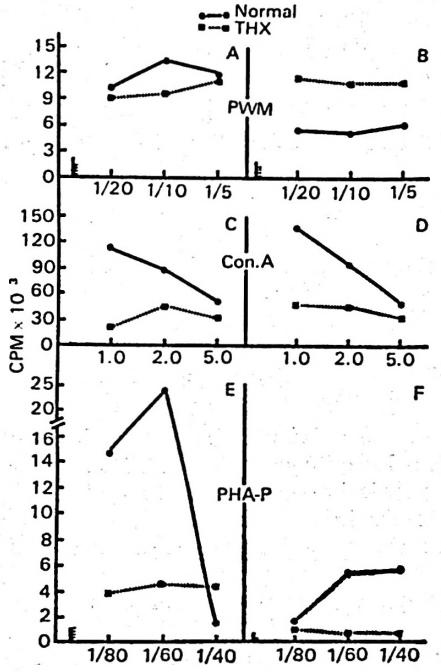

Fig. 3. *Effect of three different mitogen doses on reactivity of spleen cells from a normal and a thymectomized mouse, 11 weeks post-THX.*

A, C and E were assayed on day 2; B, D and F on day 3. The dose for each mitogen (abscissa) are as in figure 1 (see methods). Each point is the mean of three replicate cultures. The bars (lower left in each section) represent response of cultures without mitogens.

thymectomized mouse. The data are similar to those in figure 2 where only normal mice are compared. Interestingly the response to PWM also seems supressed and was also observed at 8 and 14 weeks post-THX (fig. 2).

Discussion

The non-ability of adult C57B1/6J mice to respond to PHA-P and Con A demonstrates that there is a loss of a subpopulation of peripheral T cells shortly after thymectomy. The deficiency appears to be long lasting but further experiments are necessary to clarify this. The relatively normal response to PWM suggests that bone marrow derived lymphocytes were not affected.

A large number of theta-bearing spleen cells were still present in the thymectomized mouse, an indication that a primary PHA-sensitive population of peripheral T cells (T_1 cell) is lost (11), and further suggests that these primary reactants act synergistically with the remaining T cells which also bind PHA (16). Some support for this concept comes from a recent investigation (5) showing that T cells release humoral factor(s) after reacting with PHA. These factors presumably react with adherent cells (macrophages) in the presence of PHA allowing a more efficient synthesis and release of «lymphocyte activating factors» (LAF) which have a specific stimulating effect on T cells. What is now being assessed is whether spleen cells from thymectomized mice, with and without adherent cells, can be stimulated to produce lymphocyte activating factors (LAF) and also whether active supernates of normal spleen cell cultures can support the blastogenesis of the spleen cells from thymectomized mice. A positive result would suggest that a small population of T cells is responsible for producing humoral factors which would aid a larger number of other T cells in their response to PHA.

An important recent observation is that low submitogenic doses of either PHA or Con A enhance the primary *in vitro* immune response to sheep erythrocyte antigens (13). It is known that the primary immune response to these antigens is not affected by adult thymectomy when tested in the intact mouse (11). It would appear to be a simple matter to test if spleen cells from thymectomized mice will give a normal primary *in vitro* response and then show whether submitogenic doses of PHA enhance this response. If enhancement fails it would strongly suggest that the primary PHA-sensitive T lymphocyte population is responsible, or that there are two T cell sub-population both of

which are lost after adult thymectomy. The recent observation that macrophages (adherent cells) can directly interact with PHA or Con A and release LAF (5) point to the need for clarification as to whether spleen cells from thymectomized mice can indeed produce active supernates (5) when both low and high doses of mitogens are used. If lymphocyte activating factors released by macrophages fail to stimulate blastogenesis in spleen cells from thymectomized mice it would suggest that the PHA-sensitive T cell sub-population also requires LAF, or that the LAF-sensitive T cell is distinct but similarly thymus dependent.

Another contradiction in the literature which must be clarified deals with the two observations showing on the one hand a normal primary response to sheep erythrocytes after adult thymectomy (11), whereas on the other hand it has been shown that spleen cells from similar mice do not show a primary adoptive transfer response (2). It is possible that the cell transfer system uncovers the deficiency after adult thymectomy. The T_2 cell, or mature T cell compartment, may requiere replacement by the less mature or T_1 cell (11), and this need may be magnified after cell transfer. Presumably the T_1 cell is depleted after thymectomy whereas treatment with anti-lymphocyte serum (ALS) depletes the T_2 cell (11). It should therefore be possible to restore adoptive transfer function of spleen cells from thymectomized mice (deficient in T_1) with spleen cells from ALS treated mice (deficient in T_2). Simultaneous restoration of PHA and Con A sensitivity should also be demonstrable. This problem is at present under investigation.

The response to PWM was much less supressed after adult thymectomy and is consistent with earlier studies showing specificity of this mitogen for bone marrow derived lymphocytes (17). However, it was recently demonstrated that some T cells, i.e. the cortisone resistant cells of the thymus can respond to PWM (6). Thus after simple ablation of the adult thymus loss of this population could result in a slightly depressed response to PWM; an observation consistent with our findings.

Acknowledgement

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Resumen

Se ha estudiado la respuesta blastogénica frente a los mitógenos PHA-P, Con A y PWM en los linfocitos esplénicos de ratones adultos timectomizados.

La respuesta a los mitógenos se ha determinado mediante la incorporación de timidina. Asimismo, se ha valorado los cambios producidos en los linfocitos esplénicos portadores de antígeno theta mediante anticuerpos anti-theta obtenidos de ratones AKR/S, previamente inyectados con linfocitos tímicos y esplénicos de ratones CBA/J.

Se demuestra que la respuesta frente a PHA-P y Con A decae después de la timectomía mientras que no ocurre lo mismo frente a PWM. No ha existido correlación entre la disminución de la respuesta mitogénica y los cambios observados en los linfocitos esplénicos portadores de antígeno theta.

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