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Thymic involution in pregnant mice

II. FUNCTIONAL ASPECTS OF THE REMAINING THYMOCYTES

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SUMMARY

Non-specific immunological modifications in mice during syngeneic gestation can be demonstrated. A dramatic transient thymic involution results from a significant reduction in the subpopulations of the thymic cortex with the remaining thymocytes being mainly medullary in nature. Response to PHA and Con A is greatly reduced in this pool which can normally be strongly stimulated. No suppressor cells or alteration in thymic epithelial function could be demonstrated. This unexpected low responsiveness of the remaining thymocytes was reversible after *in vitro* neuraminidase treatment. Meanwhile, MLR of thymocytes to allogeneic cells during syngeneic gestation was not impaired.

INTRODUCTION

Several studies have previously demonstrated a marked but transient involution of the thymus during pregnancy in mice (Persike, 1940; Pepper, 1961; Maroni & De Sousa, 1973; Millar, Mills & Baines, 1973). The changes in the lymphoid system during pregnancy may be part of the complete maternal modification which leads to the acceptance of the fetus. Consequently, we have studied the functions of the remaining lymphocytes in the thymus of pregnant mice, and we have shown them to be mainly of the medullary type (Le Hoang Phuc *et al.*, 1981).

During pregnancy, this significant reduction of the cortical thymocyte population appears similar to hydrocortisone-induced thymic atrophy (Ito & Hoshino, 1962) and could be linked to the increased production of steroids. To investigate if pregnancy-induced thymic atrophy was the same as the steroid effect, we compared the responsiveness of thymocytes in pregnant mice with hydrocortisone-treated ones by mitogenic (PHA and Con A) or allogeneic stimulation *in vitro*. Thymic epithelial function was tested during pregnancy by evaluation of the circulating thymic factor (facteur thymique sérique: FTS).

This study was deliberately performed in syngeneic gestations (CBA × CBA) which eliminate the immunization of the mother by paternal histocompatibility antigens (but do not exclude the intervention of embryonic or fetal antigens or of sex-linked antigens). Therefore, the alteration of the immune system can be considered as 'non-specific' (i.e. not apparently related to histocompatibility).

Abbreviations: FTS = facteur thymique sérique, HBSS = Hanks' balanced salt solution, HHC = hydrocortisone hemisuccinate, SR = steroid-resistant, MEM = minimal essential medium, MLR = mixed lymphocyte reaction, TP = thymus of pregnant mice, TV = thymus of virgin mice.

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MATERIALS AND METHODS

Mice. CBA/J mice (H-2^k) and BALB/c mice (H-2^d) were used (CSEAL, Orléans la Source, France). Pregnant mice were 6-week-old primiparous CBA/J or BALB/c females mated with males of the same strain. Six-week-old virgin female mice were used as controls.

Hydrocortisone treatment. Hydrocortisone hemisuccinate (HHC) (Roussel, Paris) was injected twice intraperitoneally (500 mg/kg) at 24-hr intervals into control or pregnant mice (during the early post-partum period). Thymuses were removed 3 days after the last injection.

Preparation of cell suspensions. Thymocytes were isolated in a Potter homogenizer. The cell suspensions were washed at 1,400 r.p.m. for 7 min and the pellet resuspended in Hanks' balanced salt solution (HBSS). Lymphoid cells were counted in trypan blue dye solution.

In vitro *stimulation by mitogens (PHA–Con A)*

Standard culture. For culture purposes, manipulations were performed under sterile conditions. Culture medium consisted of Eagle's minimal essential medium (MEM × 1) with Earle's salts (Grand Island Biological Co.) supplemented with 1% L-glutamine (200 mM), 1% non-essential amino acids (× 100), 1% sodium pyruvate (100 mM), penicillin (100 units/ml), streptomycin (100 μg/ml) and 5% heat-inactivated newborn calf serum.

Cultures were set up in microtest plates (Falcon Microtest II) with each well receiving 200 μl of medium containing 6×10^5 lymphoid cells. Phytohaemagglutinin (PHA-P, DIFCO Laboratories) and concanavalin A (Con A, Miles Yeda) were used at a concentration of 10 μg/ml.

Cultures were maintained for 48 hr at 37°C in a humidified 5% CO_2 atmosphere. One microcurie of tritiated thymidine (CEA, Saclay, France; sp. act. 1 Ci/mmol) was added to each well 20 hr before cell collection. The cultures were then harvested (Scatron multiple sample collector), diluted in scintillation fluid (PPO-POPOP, Packard), and ^{3}H-thymidine incorporation measured in a scintillation counter (Searle Delta 300). Results were expressed as differences in counts per minutes between stimulated and control cultures (Δ c.p.m.).

Suppression experiments. Control mitogen cultures (reference cultures) were set up with strongly responding steroid-resistant (SR) thymocytes from virgin mice. For suppression experiments, various numbers of cells to be tested, treated or not treated by mitomycin, were added to the reference cultures (ratios varying from 1/8 to 1/1) and stimulated by mitogens.

Neuraminidase treatment. Thymic lymphocytes (5×10^6 cells/ml) were prepared aseptically in Eagle's minimal essential medium (MEM × 1) and treated with 15 units/ml of neuraminidase (*Vibrio comma* ORKD, Behringwerke AG, Marburg/Lahn, Germany) for 30 min at 37°C. Cells were washed twice in MEM, once in culture medium containing 5% newborn calf serum and then resuspended to the appropriate concentration for culture purposes.

Control cells were manipulated at the same time without enzyme.

In vitro *stimulation by allogeneic cells*

One-way mixed lymphocyte cultures were set up between BALB/c (H-2^d) thymocytes and CBA (H-2^k) spleen cells inactivated by mitomycin C. Ten million cells/ml in culture medium were treated with 50 μg/ml of mitomycin C (Amétycine, Choay Laboratory, Paris). After a 30-min incubation period at 37°C, cells were washed three times; the pellet was resuspended in culture medium with 10% human AB serum (10×10^6 cells/ml).

One million thymic responder cells were mixed with 1×10^6 mitomycin-inactivated allogeneic spleen cells in a total volume of 200 μl per well of microtest plate. For control cultures, 1×10^6 thymic BALB/c (H-2^d) responder cells were mixed with 1×10^6 mitomycin-inactivated syngeneic spleen cells.

One microcurie of tritiated thymidine was added to each well after various culture periods (48, 72 or 96 hr) in a humidified 5% CO_2 atmosphere. The cultures were harvested 20 hr later and the radioactivity was counted as described above. Results are given as stimulation index:

$$\text{SI} = \frac{\text{c.p.m. in stimulated culture}}{\text{c.p.m. in control culture}}.$$

Evaluation of the levels of circulating thymic factor (FTS) during pregnancy

The method for FTS evaluation has already been reported in detail (Bach *et al.*, 1975; Dardenne & Bach, 1975). In brief, it consists of filtering the serum sample through an Amicon CF50 membrane, which removes molecules with a molecular weight higher than 50,000 daltons, and of testing the capacity of the filtrate to confer sensitivity to azathioprine to the spleen rosette-forming cells (RFC) from adult thymectomized mice. Results are expressed as the maximal serum dilution giving a significant azathioprine conversion.

RESULTS

Response of thymic cells to PHA and Con A

PHA and Con A responses were tested on the total thymocyte population of pregnant mice, during the period of maximum thymic atrophy (between day 20 of gestation and day 6 of the post-partum period), and of virgin mice. Control thymocytes showed a very low response to both PHA and to Con A. This poor response of normal adult thymocytes to phytomitogen is predictable since the medullary cells, which are the responding cells, represent less than 5% of the total cell population. In testing the thymocytes of pregnant mice, we found an almost negative response to mitogens (Table 1).

In order to test only the SR thymocytes and to eliminate the cortical lymphocytes which may persist in the thymus of pregnant mice, we treated pregnant mice (during the first week post-partum) and virgin mice with HHC. *In vivo* treatment of virgin adult mice by hydrocortisone enhanced the response of thymic cells to PHA and Con A due to the destruction of cortical non-competent cells (Stobo, 1972) (Table 1). The thymocyte response of hydrocortisone-treated pregnant mice was also enhanced but remained much lower than the response of thymocytes from hydrocortisone-treated virgin mice (Table 1).

These results are paradoxical since the thymocytes remaining during gestation were mainly of medullary origin which are usually highly responsive to mitogens. Among many, two hypotheses can readily be tested to explain this phenomenon: either a population of suppressive cells develops in the thymus during gestation or thymocyte membrane receptors to mitogens are masked during pregnancy.

Table 1. Stimulation by PHA and Con A of SR or total thymus cell population from virgin and pregnant mice

	PHA*	Con A*
Total thymocyte population		
Virgin mice†	0·3 ± 0·1	2·8 ± 0·5
Pregnant mice†	0·05 ± 0·01	0·2 ± 0·1
P value	< 0·01	< 0·01
SR thymocyte population		
Virgin mice‡	82·6 ± 3·0	154·0 ± 34·2
Pregnant mice‡	18·5 ± 3·8	19·2 ± 2·5
P value	< 0·01	< 0·02

Total thymocyte populations were tested between day 10 of gestation and the first week of the post-partum period. SR thymocytes were tested during the first week of the post-partum period (HHC injection given 2–3 days post-delivery). Response of pregnant mouse thymocytes is significantly lower than that of virgin mouse thymocytes.

* c.p.m. $\times 10^{-3}$.

† Mean value ± s.e.m. for seven experiments.

‡ Mean value ± s.e.m. for four experiments.

Eight thymuses were pooled for each experiment.

Table 2. Test for suppressive activity in thymocytes from pregnant mice: co-culture between SR thymocytes from virgin mice and total or mitomycin-treated SR thymocytes from virgin or pregnant mice

Responding cells (SR thymocytes of virgin mice)	Cells added	PHA*	Con A*
Expt 1			
6×10^5	0	58·7	166·8
6×10^5	6×10^5 total thymocytes of virgin mice	47·7	149·9
6×10^5	6×10^5 total thymocytes of pregnant mice†	56·9	156·2
Expt 2			
6×10^5	0	68·0	130·4
6×10^5	6×10^5 SR mitomycin-treated thymocytes of virgin mice	68·0	119·3
6×10^5	6×10^5 SR mitomycin-treated thymocytes of pregnant mice‡	67·4	119·1

No suppression could be demonstrated with either the total thymocyte population or with the SR mitomycin-treated thymocytes.

* c.p.m. $\times 10^{-3}$.

† Five thymuses from day 1 post-partum.

‡ Fifteen thymuses from day 3 post-partum.

Suppression experiments: effect of thymocytes from pregnant mice on the mitogenic response of (SR) thymocytes

To assess the presence of suppressive cells in the thymus of pregnant mice, thymocytes from virgin and pregnant mice were thoroughly washed before suppression experiments to avoid, as far as possible, carry-over of pregnancy-induced suppressive serum factors. Under these conditions no suppression was seen in any experiment when the total thymocyte population from pregnant mice was added to stimulated cultures of medullary thymocytes from virgin mice (Table 2).

The same observation was noted in experiments comparing the response to mitogens of the co-cultures set up between SR thymocytes of pregnant mice and SR thymocytes of virgin mice and the sum of responses of each of the two thymocyte populations cultured alone (Table 3). By these

Table 3. Test for suppressive activity in thymocytes from pregnant mice: co-culture between non-mitomycin-treated SR thymocytes from pregnant and virgin mice.

	3×10^5 SR thymocytes of virgin mice (A)	3×10^5 SR thymocytes of pregnant mice (B)	A+B	A+A
Expt 1				
PHA	8·5	2·7	14·2	18·6
Con A	44·5	7·3	60·8	98·4
Expt 2				
PHA	17·8	3·0	25·4	31·8
Con A	57·8	7·5	85·9	124·5

Thymocytes of group B do not contain any suppressive activity, as results of A+B co-cultures are nearly equal to the cumulative results of A and B cultures alone.

Table 4. Effect of neuraminidase on the mitogenic response of thymocytes from virgin and pregnant mice

	Neuraminidase treatment	Total thymocyte population of virgin mice	SR thymocyte population of virgin mice	SR thymocyte population of pregnant mice
Expt 1*				
PHA	–	1·5	30·9	2·9
	+	1·4	30·9	19·2
Con A	–	3·7	128·3	6·7
	+	5·6	105·0	82·5
Expt 2*				
PHA	–	1·9	85·5	14·7
	+	1·5	81·1	30·2
Con A	–	3·8	208·8	23·8
	+	4·8	209·1	87·3
Expt 3†				
PHA	–	2·1	69·1	39·9
	+	1·7	64·3	38·0
Con A	–	7·4	163·2	120·5
	+	7·8	159·1	116·0

Thymocytes from normal or HHC-treated mice were incubated *in vitro* with neuraminidase and cultured with PHA or Con A. Results are given in c.p.m. $\times 10^{-3}$. Neuraminidase pretreatment of thymic cells induced an increase in PHA and Con A reactivity by pregnant mice, without affecting the reactivity of virgin mice (Expt 1 and 2). Once the thymocytes had recovered a subnormal response (day 19 post-partum), neuraminidase pretreatment of the cells was ineffective (Expt 3).

* Thymuses from day 3 post-partum.

† Thymuses from day 19 post-partum.

methods, it was impossible to demonstrate the presence of suppressive cells in the thymus of pregnant mice.

Effect of neuraminidase treatment on the mitogenic response of thymocytes from pregnant mice

In order to unmask hidden mitogen membrane receptors, the SR thymocyte population from pregnant mice was treated with neuraminidase before initiating the *in vitro* mitogenic stimulation cultures.

When SR thymocytes were pretreated with neuraminidase, an increase in the proliferative response to PHA and Con A was obtained with the thymocytes of pregnant mice tested during the period of maximal thymic atrophy but not with the control thymocytes from virgin mice (Table 4, experiments 1 and 2). The increase in the proliferative response was relatively stronger with Con A than with PHA.

In the experiments performed during the period of cellular recovery for the thymus of pregnant mice, when the thymocytes had already recovered a subnormal response to mitogens, neuraminidase pretreatment did not increase the proliferative response (Table 4, experiment 3). Thus it appears that the relative unresponsiveness of the thymocytes of pregnant mice can be related to a masking of the active sites on the thymocyte membrane.

MLR between thymocytes from pregnant mice and allogeneic or syngeneic spleen cells

The proliferative reactivity of thymocytes from pregnant mice induced by allogeneic stimulation was investigated in order to determine if the same low responsiveness occurs in MLR as with mitogen stimulation.

Table 5. MLR of total or SR thymocyte populations from virgin and pregnant mice

	Day of culture		
Responder cells	3	4	5
Total thymocyte population			
Virgin mice†	3·9 ± 1·0*	4·4 ± 1·6	2·2 ± 0·6
Pregnant mice‡	24·9 ± 7·8	26·9 ± 6·6	24·5 ± 9·5
P value	< 0·01	< 0·01	< 0·01
SR thymocyte population			
Virgin mice§	16·1 ± 9·5	14·6 ± 5·1	n.d.
Pregnant mice§	16·9 ± 1·3	11·5 ± 1·6	n.d.
P value	n.s.	n.s.	

Pregnant mice were tested during the first week of the post-partum period. At all culture durations (days 3, 4 and 5) the pregnant mice exhibited an increased MLR compared to the virgin mice ($P < 0·01$). SR thymocyte populations in pregnant and virgin mice were equivalent.

* Stimulation index.

† Mean value ± s.e.m. for 14 experiments.

‡ Mean value ± s.e.m. for eight experiments.

§ Mean value ± s.e.m. for three experiments.

Responder cells were thymocytes from primiparous syngeneically pregnant BALB/c mice (H-2^d) killed between day 20 of gestation and day 6 post-partum. Virgin females of the same age were used as controls. Stimulator cells were splenic lymphocytes from normal CBA/J mice (H-2^k). Using total thymocyte populations, the kinetic study of the reaction showed that the thymocytes from pregnant mice have a much higher proliferative response than the thymocytes from control virgin mice either at day 3, 4 or 5 of the culture (Table 5). The pregnancy-induced increase in the MLR response appears to be due to the concentration of medullary immunocompetent cells in the thymus of pregnant mice as a result of the involution of the thymic cortex.

In another set of experiments, responder thymocytes from hydrocortisone-treated virgin and pregnant mice were used. Following 3 or 4 days of incubation, SR thymocytes from virgin or pregnant mice did not display any significant difference in their reactivity (Table 5).

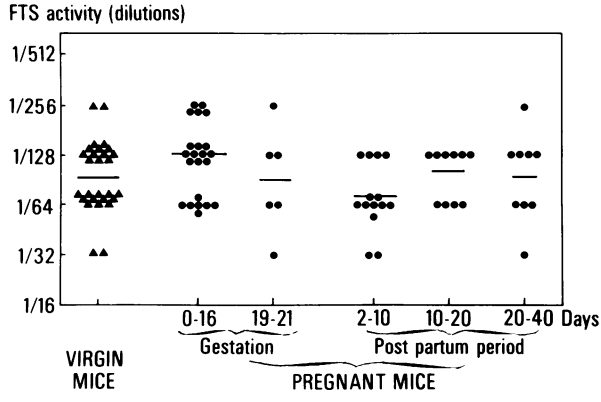

Fig. 1. FTS serum activity during pregnancy. Results are expressed as the maximal dilution of the sample giving rosette inhibition of more than 50%. There was no difference in FTS activity between virgin controls and pregnant mice, whatever the stage of pregnancy.

Thus in terms of the MLR, the total thymocyte population of untreated pregnant mice is similar to the SR thymocyte population of HHC-treated virgin mice.

Evaluation of the level of circulating thymic factor (FTS) during gestation

FTS activity was evaluated in primiparous pregnant CBA/J mice (Fig. 1). The thymic activity remained constant during the course of the pregnancy and the post-partum period, at the same level (1/64–1/128) as obtained in control virgin mice of the same age. The epithelial function appeared to be unaltered during the thymic involution in pregnant mice.

DISCUSSION

In our previous studies, we have demonstrated that there is a significant reduction in the cortical thymocyte subpopulation during gestation in mice. Thus the involution of the thymus in pregnant mice can be compared with the hydrocortisone-induced thymic atrophy in virgin mice (Le Hoang Phuc *et al.*, 1981). Indeed, there is increased steroid production during gestation. The question is whether or not during pregnancy the atrophic thymus can be functionally compared to the thymus of exogenously HHC-treated mice.

If the physiological model of gestation is analogous to the experimental model of hydrocortisone treatment, the T cell mitogen response by the total thymocyte population of pregnant mice is expected to be strong like that of the SR thymocyte population from virgin mice. On the contrary, we found that the total thymocyte population of pregnant mice is even less responsive than that of virgin mice, whereas the SR thymocyte population of virgin mice responded strongly as was anticipated (Table 1) (Blomgren & Andersson, 1971; Blomgren & Svedmyr, 1971; Stobo, 1972). Anderson (1978) found a weak increase in the PHA response of the total thymocyte population during pregnancy. However, this study does not investigate the only SR population and was done on an outbred strain of mice (Swiss) and with different culture procedure.

Gravid females were treated with exogenous hydrocortisone, thereby avoiding the interference of the small remaining cortical subpopulation and allowing comparison of only the medullary SR subpopulations. Under these conditions, the response to mitogens increased in the hydrocortisone-treated pregnant mice but did not reach the high levels seen in the hydrocortisone-treated virgin mice (Table 1). Therefore, the gestational thymus is not strictly analogous to the hydrocortisone-treated model.

Hormonal effect on cellular metabolism can play a transient and reversible role. *In vitro* studies show that the dose of the steroids and the incubation time are critical for the inhibition of the lymphocyte response to mitogens. To suppress the mitogen response of lymphocytes *in vitro*, high concentrations of steroids (glucocorticoids or sex steroids) are needed, and the longer the duration of the incubation the stronger the inhibition (Ablin *et al.*, 1974; Homo, Dardenne & Duval, 1981; Mendelsohn, Multer & Bernheim, 1977; Schiff, Mercier & Buckley, 1975; Wyle & Kent, 1977). Mice treated with two intraperitoneal injections of HHC display a rapid and transient increase in the level of circulating cortisone, whereas during gestation, there is a continuous and prolonged impregnation by steroids including cortisol, oestrogens, progesterone and chorionic gonadotrophins. Exogenous hydrocortisone treatment destroys the cortex without affecting medullary competence, while the long-lasting infiltration by multiple endogenous hormones during gestation may alter medullary cell functions and induce an inhibition of the proliferative mitogenic response. This prolonged contact with steroids may account for the very slow recovery of the mitogenic response capability by the remaining number of thymocytes during the post-partum period (3 weeks) as compared with the more rapid cell recovery following exogenous steroid treatment (Papiernik & Bach, 1977). Indeed, we could not find in co-culture experiments (Table 2) enrichment of a suppressive population which could explain such a reduction in the mitogenic response in pregnant mice. Functional depression of the thymocytes could not be explained either by an absence of lymphocyte maturation due to an FTS hyposecretion, since normal levels of FTS activity were found throughout gestation and the post-partum period (Fig. 1).

During pregnancy, a modification of the mitogen membrane receptors on thymocytes may also

explain the low proliferative response. One can imagine specific mitogen membrane receptors, either masked by a soluble factor or hidden like cryptic sites, preventing the binding of lectin to the cells and the resulting proliferative response. To investigate this possibility, we treated thymocytes of pregnant mice with neuraminidase to expose membrane receptors to mitogens as was done by Currie, Van Doorninck & Bagshawe (1968) to unmask surface transplantation antigens on early mouse trophoblast. Neuraminidase removes cell surface sialic acid residues and has many non-specific effects such as unmasking surface antigens, altering the charge of the plasma membrane and exposing cryptic antigens or receptors for immunoglobulin (Bagshawe & Currie, 1968; Galili & Schlesinger, 1974; Grothaus *et al.*, 1971; Haegert, 1979; Rosenberg, Plocinik & Rogentine, 1972).

In vitro neuraminidase treatment of the thymocytes from pregnant mice prior to culture enabled us to obtain a higher almost normal response when the SR medullary thymocyte subpopulation was tested (Table 4). A soluble factor, which masks the mitogen membrane receptors, may be either inactivated or released into the medium by this procedure. This soluble factor may be analogous to α-fetoprotein which has neuraminidase-sensitive suppressive activities (Zimmerman, Voorting-Hawking & Michael, 1977).

The proliferative response to allogeneic stimulation of thymocytes from pregnant mice was not impaired. The higher response observed in pregnant mice compared with virgin mice was exactly the one we would expect after concentration of the thymic medullary immunocompetent cells (Blomgren & Svedmyr, 1971) (Table 5). This was demonstrated by experiments showing identical MLR obtained with thymocytes from hydrocortisone-treated pregnant or virgin mice (Table 5). Thus impairment of mitogenic response to PHA and Con A is not associated with alterations of the mitogenic response to allogeneic cells. This discrepancy may be explained by a specific blocking of mitogen membrane receptors without modification of an allogeneic recognition receptor.

T lymphocyte subpopulations responding to mitogens are known not to be identical to T lymphocyte subpopulations reacting in MLR (Colley, Shih Wu & Waksman, 1970; Knight, Newey & Ling, 1973; Elliot, 1977). Hence, one can suppose that only one of these two types of cells is modified by pregnancy. Further studies are needed to analyse the precise role and the mechanisms of thymic involution in pregnant mice, since these non-specific immunological changes of the thymus during gestation may be part of the overall phenomenon leading to fetus acceptance in mammals.

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