Immunology, 1974, **27**, 299.

Studies on Thymus Products

III. EPITHELIAL ORIGIN OF THE SERUM THYMIC FACTOR

MIREILLE DARDENNE,* MARTINE PAPIERNIK,* J.-F. BACH* AND O. STUTMAN

* *Hôpital Necker, INSERM U* 25, 161, *rue de Sèvres–*75730*–Paris–Cedex* 15, *and*
† *Memorial Sloan Kettering Cancer Center, New York City, New York* 10021, *U.S.A.*

(*Received* 17*th October* 1973*; accepted for publication* 6*th November* 1973)

Summary. Serum thymic factor (TF) has been tested by its action on spleen rosette-forming cells from adult thymectomized mice. It has been confirmed in a blind study using coded serum samples that TF disappeared early after adult thymectomy and reappeared after grafting a thymus, either as a free graft or enclosed in cell impermeable diffusion chambers. Similar reconstitution was also obtained by grafting a non-lymphoid epithelial thymoma or pure epithelial thymus, obtained by *in vivo* incubation of a thymus within a diffusion chamber in an intermediate host. Conversely, TF levels were not restored in thymectomized animals treated with dispersed spleen cells or with dispersed thymic lymphocytes.

INTRODUCTION

We have previously reported the presence in normal mouse serum of a factor (or hormone) produced by the thymus (Bach and Dardenne, 1972, 1973). This thymic factor (TF) disappears from serum within a few hours after thymectomy and reappears in thymectomized mice after thymus grafting or injection of thymic extracts (Bach and Dardenne, 1972, 1973). The present report confirms the thymic origin of TF with coded experiments performed by two different laboratories, one of which prepared the sera for testing while the other tested the TF activity of those sera. The absolute agreement between presence of thymic function in the experimental animals and detectable level of TF in their sera clearly supports the contention of the thymic origin of TF. The present report also shows that the TF-secreting cells in thymus are probably the reticuloepithelial cells. Significant reappearance of serum TF was obtained in thymectomized mice after grafting non-lymphoid functional thymomas (Stutman, Yunis and Good, 1969a) or after grafting a thymus that had become essentially epithelial after a 6–8 day stay in a diffusion chamber in an intermediate host (Hays, 1969).

MATERIALS AND METHODS

Mice

C3Hf/Umc from the mouse colonies of the Memorial-Sloan Kettering Cancer Center and C57/Bl6 mice from Charles Rivers were used in all the experiments.

Correspondence: Dr M. Dardenne, Hôpital Necker, 161, rue de Sèvres 75730–Paris–Cedex 15, France.

Thymectomy

Thymectomies were performed by suction in 6-day-old C3Hf/Umc or 6–9-week-old C57/Bl6 mice as described in previous publications (Stutman *et al.*, 1969a; Bach and Dardenne, 1972).

Thymus grafting

One thymic lobe from syngeneic 20-day-old C3Hf/Umc mice or one thymic lobe within a cell impermeable diffusion chamber prepared with Millipore filters of 0·22 μm mean pore size, sealed to lucite rings, were implanted intraperitoneally, at 20 days of age, into the thymectomized C3Hf/Umc hosts. Intraperitoneal grafting as well as construction of the diffusion chambers were performed as described in previous publications (Stutman *et al.*, 1969a, b).

Epithelial thymoma

Thymomas were induced by intrathymic application at birth of 0·1 mg of 7-12-dimethylbenzanthracene in C3H/Umc mice. When used in the present experiments the thymoma (Thy. number 2) was in its fifth transplant generation. The thymectomized C3Hf/Umc mice were grafted subcutaneously with 1×10^6 thymoma cells. Transplantation procedures and production of tumour cell suspensions have been described in previous publications (Stutman *et al.*, 1969a, b). Description of the morphological and functional characteristics of the non-lymphoid thymomas has been reported previously (Stutman *et al.*, 1968, 1969a). The non-lymphoid reticuloepithelial nature of the thymoma, as well as the probable secretory nature of some of its cells, has been described at the ultrastructural level (Bockman and Stutman, 1969).

Epithelial thymus

Two thymus lobes from newborn C57/Bl6 mice were placed in a Millipore chamber prepared with filters of 0·45 μm mean pore size and sealed with acetone to lucite rings (Hays, 1969). The chambers were placed intraperitoneally into adult C57/Bl6 mice. Six to 8 days later, chambers were recovered, the thymus were removed from the chambers and regrafted intraperitoneally into adult thymectomized C57/Bl6 mice. The epithelial nature of the thymus within the chambers is illustrated in Fig. 1b.

Serum TF determination

Serum TF determination was performed as already described (Bach and Dardenne, 1972, 1973). In brief, sera were filtered on CF-50 Amicon filters and tested for their ability to confer on spleen rosette-forming cells from adult thymectomized C57/Bl6 mice the sensitivity to azathioprine they otherwise lacked. In some experiments the UM2 Amicon membranes were also used (molecular weight cut-off: 1000).

Experimental design

Two sets of experiments were performed.

(1) Six-day-old thymectomized C3Hf/Umc mice were: (a) left untreated; (b) grafted intraperitoneally with one syngeneic thymic lobe; (c) grafted intraperitoneally with a thymic lobe enclosed in a diffusion chamber; (d) grafted subcutaneously with 1×10^6 cells from C3H/Umc thymoma number 2, a functional epithelial thymoma; (e) injected intraperitoneally with 100×10^6 syngeneic spleen cells from 3–4-month-old C3Hf/Umc

donors. An additional sixth group consisted of sham-thymectomized mice left untreated. All the treatments from (b) to (e), were performed when the thymectomized animals were 20 days old. At 30 days of age, all the animals were bled, and the sera from each group were pooled (five to six animals per group), coded and assayed for TF. When the assays were performed the codes were broken and the results correlated with each experimental group.

(2) Two thymus lobes from new born C57/Bl6 mice were placed in a diffusion chamber which was grafted intraperitoneally into normal adult C57/Bl6 mice. Six to 8 days later the chamber was collected, the thymus was removed from the chamber, and regrafted intraperitoneally into an adult thymectomized mouse. In a few cases, after being removed from the chamber and examined, the thymus was put back into a new Millipore chamber and the chamber was placed intraperitoneally into an adult thymectomized recipient.

RESULTS

EFFECTS OF THYMUS ABLATION AND REPLACEMENT ON SERUM TF LEVELS

Table 1 shows the effect of different treatments on the TF serum levels of C3Hf/Umc mice thymectomized at 6 days of age and subsequently left intact or treated at 20 days of

TABLE 1

THYMIC EPITHELIAL ORIGIN OF SERUM THYMIC FACTOR (TF) IN MICE

Experimental group	Operation	Treatment	Serum TF
1	Thymectomy at 6 days of age	None	1/4
2	Thymectomy at 6 days of age	One thymic lobe, intraperitoneally	1/128
3	Thymectomy at 6 days of age	One thymic lobe, i.p. in diffusion chamber	1/16
4	Thymectomy at 6 days of age	1×10^6 cells from C3H thymoma, subcutaneously	1/32
5	Thymectomy at 6 days of age	100×10^6 adult syngeneic spleen, i.p.	1/4
6	Sham-thymectomy at 6 days of age	None	1/128

All the mice were C3Hf/Umc, all the treatments were performed at 20 days of age, and the serum samples (pooled from five to six animals per group) were obtained at 30 days of age (i.e. 10 days after treatment). For details on treatment procedures, donors, diffusion chamber construction, thymomas and TF determinations, see Materials and Methods section. Serum TF values represent the average of three determinations.

age with one thymus lobe as an intraperitoneal free graft. In previous experiments we have shown that diffusion chambers prepared with filters of 0·22 μm mean pore size were truly impermeable to the passage of cells (Stutman *et al.*, 1969a). Confirming our previous results we found that before thymus grafting, no detectable levels of serum TF could be detected (in this particular experiment, 24 days after thymectomy at 6 days of age, Group 1, Table 1). Conversely, grafting of a thymus as a free graft restored the TF levels to detectable levels (Group 2, Table 1). Thymus grafting produced a recovery comparable to the values detected in sham-thymectomized controls (Group 6, Table 1). Table 1 (group 5)

also shows that the injection of 100×10^6 adult syngeneic spleen cells did not restore TF serum levels in the thymectomized animals, these last results suggesting the thymus dependency of TF levels in serum. It should be stressed that from a functional standpoint, the injection of adult spleen cells produces adoptive immune restoration of the thymectomized hosts, which is exclusively mediated by donor cells (Stutman *et al.*, 1969b). The very low level of TF in the serum of the thymectomized animals was subsequently

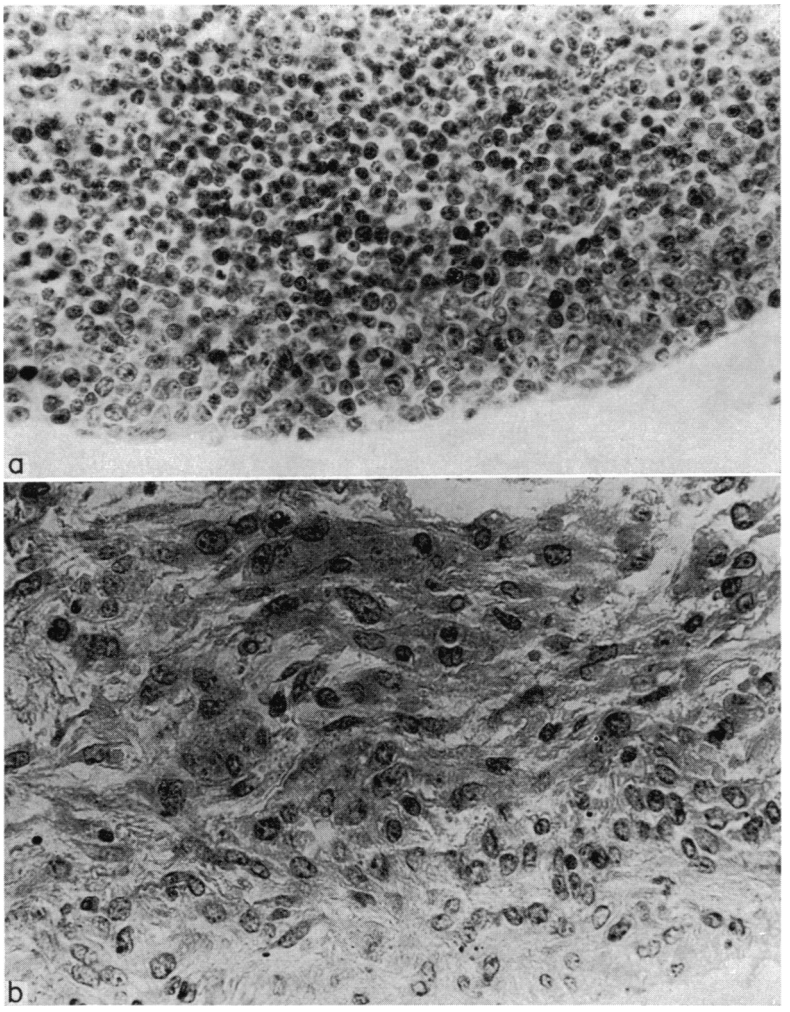

FIG. 1. Thymus histology (a) before and (b) after 7 days in an intraperitoneal diffusion chamber.

verified by further concentration of the Amicon CF50 filtrates on UM2 membranes; this procedure raised the TF level from sham-thymectomized mice to 1/4000, whereas the TF level from the thymectomized mice remained less than 1/4.

EFFECT OF THYMUS GRAFTING IN DIFFUSION CHAMBERS ON SERUM TF LEVELS

Restoration of TF levels in thymectomized mice was also obtained by grafting a thymus

enclosed in a cell impermeable diffusion chamber (Group 3, Table 1). However, although TF levels were increased, the restoration was much less dramatic than with a free thymus graft.

EFFECT OF NON-LYMPHOID THYMOMAS ON SERUM TF LEVELS

Table 1 shows (group 4) that the subcutaneous grafting of 1×10^6 tumour cells derived from a functional non-lymphoid epithelial thymoma resulted in a significant recovery of serum TF levels. The subcutaneous injection of an identical number of normal adult thymocytes in suspension did not produce detectable levels of serum TF in the thymectomized hosts (not included in Table 1).

EFFECT OF EPITHELIAL THYMUS GRAFTING ON SERUM TF LEVELS

Epithelial thymuses were obtained by *in vivo* incubation of two thymic lobes in diffusion chambers in an intermediate host (Hays, 1969). The absence of detectable lymphocytes

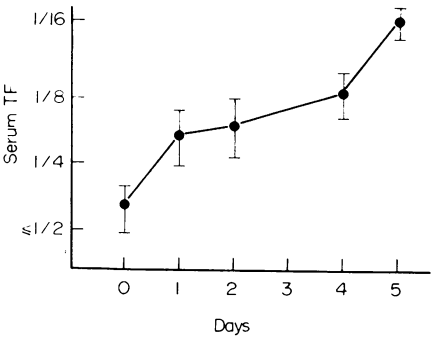

FIG. 2. Serum TF levels after grafting adult thymectomized C57/Bl6 mice with an epithelial thymus obtained after *in vivo* incubation of a whole thymus within a diffusion chamber grafted intraperitoneally in an intermediate syngeneic host (mean $\pm$ 2 s.d.).

was verified histologically (Fig. 1b). Whether the epithelial thymus was grafted directly into adult thymectomized mice or within a diffusion chamber, such epithelial thymus grafts partially restored TF levels, showing significant restoration 2 days after grafting (Fig. 2).

DISCUSSION

The thymic origin of the factor assayed in the rosette test which restores to spleen rosette-forming cells from adult thymectomized mice the normal sensitivity to azathioprine which they lack, was confirmed in the present 'blind' study using coded serum samples derived from mice in which the thymus was either ablated or replaced by free grafts or by grafts enclosed in diffusion chambers. It must be emphasized that no significant TF has ever been found in thymectomized or nude mice, even after concentration of the serum (Bach and Dardenne, 1973).

The epithelial origin of TF might be suspected in view of the secretory activity of the normal thymic epithelial cells demonstrated in electron microscopy (Clark, 1968).

Results obtained with young NZB mice, showing absence of TF in serum (Bach, Dardenne and Salomon, 1973) are also suggestive, since the NZB mice present an early atrophy of the thymus epithelium (De Vries and Hijmans, 1967). Similarly, epithelial cells appear first in the foetal thymus before lymphoid cells (Owen, 1972), and epithelial thymus grafts are rapidly repopulated by lymphocytes, whether epithelial grafts are obtained from mouse embryos (Biggar, Stutman and Good, 1972), from *in vitro* culture (Mandel, Russell and Byrd, 1972) or after *in vivo* incubation in diffusion chambers in an intermediate host (Hays, 1969), as was used here. The epithelial origin of TF is demonstrated here by the significant reconstitution of TF serum levels in thymectomized mice by grafting epithelial cells in relatively small numbers and by the effects observed with non-lymphoid reticuloepithelial thymomas. One may note that the grafting of crude suspensions of thymocytes, which include mainly the lymphoid component, does not afford such reconstitution.

These results support a hypothesis according to which the thymic epithelial stroma is colonized by haemopoietic cells which differentiate into T-cell precursors and from which the peripheral T cells originate (Ford, 1966; Stutman, 1970). Besides this central action on stem cells, the thymic factor secreted by epithelial cells probably contributes to the maturation of post-thymic cells in the periphery (Bach and Dardenne, 1972, 1973; Stutman, Yunis and Good, 1970).

ACKNOWLEDGMENTS

This work was supported by INSERM, C.N.R.S., D.G.R.S.T. and the U.S. Public Health Service grants numbers CA-12865 and CA-08748 and PR71 from the American Cancer Society.

REFERENCES

Bach, J. F. and Dardenne, M. (1972). 'Thymus dependency of rosette forming cells. Evidence for a circulating thymic hormone.' *Transplant. Proc.*, **4,** 345.

Bach, J. F. and Dardenne, M. (1973). 'Studies on thymus products. II. Demonstration and characterization of a circulating thymic hormone.' *Immunology*, **25,** 353.

Bach, J. F., Dardenne, M. and Salomon, J. C. (1973). 'Studies on thymus products. IV. Absence of serum thymic activity in adult NZB and B/W mice.' *Clin. exp. Immunol.*, **14,** 247.

Biggar, W. D., Stutman, O. and Good, R. A. (1972). 'Morphological and functional studies of fetal thymus transplants in mice.' *J. exp. Med.*, **135,** 793.

Bockman, D. E. and Stutman, O. (1969). 'Fine structure of a transplanted chemically induced non-lymphoid thymoma.' *Cancer Res.*, **29,** 1663.

Clark, S. L. (1968). 'Incorporation of sulfate by the mouse thymus: its relation to secretion by medullary epithelial cells and to thymic lymphopoiesis.' *J. exp. Med.*, **128,** 920.

De Vries, M. J. and Hijmans, W. (1967). 'Pathological changes of thymic epithelial cells and autoimmune diseases in NZB, NZW, and (NZB × NZW) mice.' *Immunology*, **12,** 179.

Ford, C. E. (1966). 'Traffic of lymphoid cells in the body.' *CIBA Symposium on The Thymus: Experimental and Clinical Studies* (Ed. by G. E. W. Wolstenholme and R. Porter), p. 131. Little-Brown, Boston.

Hays, E. F. (1969). 'The effect of epithelial remnant and whole organ grafts of thymus on the recovery of thymectomized irradiated mice.' *J. exp. Med.*, **129,** 1235.

Mandel, T., Russel, P. J. and Byrd, W. (1972). 'Differentiation of the thymus *in vivo* and *in vitro*.' *Cell Interaction, Third Lepetit Colloquium* (Ed. by L. G. Silvestri), p. 183. North-Holland, Amsterdam.

Osoba, D. and Miller, J. F. A. P. (1963). 'Evidence for humoral thymus factor responsible for the maturation of immunological faculty.' *Nature (Lond.)*, **199,** 653.

Owen, J. J. T. (1972). 'The origins and development of lymphocyte populations.' *Ontogeny of Acquired Immunity*, p. 35. North-Holland, Amsterdam.

Stutman, O., Yunis, E. J. and Good, R. A. (1968). 'Carcinogen-induced tumors of the thymus. I. Restoration of neonatally thymectomized mice with a functional thymoma.' *J. nat. Cancer Inst.*, **41,** 1431.

Stutman, O., Yunis, E. J. and Good, R. A. (1969a). 'Carcinogen-induced tumors of the thymus. III. Restoration of neonatally thymectomized mice with thymomas in cell impermeable chambers.' *J. nat. Cancer Inst.*, **43,** 499.

Stutman, O., Yunis, E. J. and Good, R. A. (1969b). 'Carcinogen-induced tumors of the thymus. IV. Humoral influences of normal thymus and functional thymomas and influence of post thymectomy period on restoration.' *J. exp. Med.*, **130,** 809.

Stutman, O. (1970). 'Hemopoietic origin of cells responding to phytohemagglutinin in mouse lymph nodes.' *Proceedings of the 5th Leukocyte Culture Conference* (Ed. by J. Harris), p. 671. Academic Press, New York.

Stutman, O., Yunis, E. J. and Good, R. A. (1970). 'Studies on thymus function. II. Cooperative effect of newborn and embryonic hemopoietic liver cells with thymus function.' *J. exp. Med.*, **132,** 601.