STUDIES ON SOME PHYSICOCHEMICAL PROPERTIES OF A THYMUS HUMORAL FACTOR CONFERRING IMMUNOCOMPETENCE ON LYMPHOID CELLS

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A humoral component of the thymus appears to be involved in the process leading to attainment of immunological competence by lymphoid cells (1–4). In previous experiments we have demonstrated that this action of a thymus humoral factor can be exerted directly upon dissociated cells from lymphoid tissue. Spleen cells from neonatally thymectomized mice exposed to extracts prepared from thymus of syngeneic origin acquired the capacity to initiate a graft-*versus*-host reaction against F_1 spleen explants (5). This assay seemed to us a useful tool to answer further questions related to the chemical nature of the thymic factor and to the mechanism of its interaction with target cells. The present communication describes experiments aimed towards characterization of some physicochemical properties of the active agent of thymus extracts and investigation of the effects of these preparations when injected into normal and thymectomized mice. In order to obtain larger quantities of material for fractionation, xenogeneic thymus extract was required. Thus it was first necessary to determine that direct in vitro induction of immune competence of lymphoid cells can be achieved by thymus extracts of nonsyngeneic origin, and the activity of xenogeneic as well as allogeneic preparations on spleen cells from neonatally thymectomized mice was assayed. Fractions prepared from xenogeneic extracts by prolonged centrifugation, dialysis, and ultrafiltration were then assayed in the in vitro test of graft-*versus*-host reactivity. In addition some of the active preparations were injected into neonatally thymectomized or intact mice and the immunocompetence of spleen cells of these mice was evaluated either by the in vitro graft-*versus*-host reaction (6) or by the in vivo Simonsen assay (7).

Materials and Methods

Experimental Animals.—C57BL/6 mice originally obtained from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and bred at the Weizmann Institute by sibling mating and (C3H/eb x C57BL/6)F_1 mice were used in these experiments. The animals were

kept at 22°–26°C and fed Purina Laboratory Chow pellets and tap water *ad libitum* and weaned at 6 wk of age.

C57Bl/6 mice, the source of the cells tested in these experiments, were thymectomized within 24 hr after birth by a modification of Miller's technique (8). Any operated animal found to contain a thymic remnant was discarded from the experiment. (C3H/eb x C57BL/6) F_1 mice served as the host animals for the in vivo test of graft-*versus*-host reactivity and the source of spleen explants for the in vitro graft-*versus*-host assay.

Preparation of Extracts.—Allogeneic thymus and spleen extracts were prepared from inbred C3H/eb, SWR/Jax, or (C3H/eb x C57BL/6)F_1 mice by a method described previously (5). Extracts of calf thymus and spleen were prepared from young animals obtained from a local slaughterhouse. After removal of the capsule and blood vessels, tissues were mixed with 0.1 M sodium phosphate buffer, pH 7.4 (w/v 1:2) and disintegrated in a Virtis homogenizer (The Virtis Company, Inc., Gardiner, N.Y.). The crude homogenates were centrifuged at 2500 *g* for 20 min and the resulting supernatant centrifuged at 100,000 *g* for 1 or 5 hr (as indicated in Results) in a Beckman Model L preparative ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The supernatant was strained through gauze and the extract diluted to contain 10 mg protein/ml as determined by the biuret reaction. All procedures were carried out at 0°–5°C with aseptic precautions. After sterilization through Millipore filters of 0.45 μ, porosity, extracts were stored at −10°C.

Extracts of calf thymus or spleen (25 ml of 100,000 *g* supernatant) were dialyzed in a cellophane dialysis sac No. 27/32 (Union Carbide Corporation, Chicago, Ill.) for 60 hr with stirring at 5°C against 500 ml of 0.005 M Na phosphate buffer, pH 7.4. This routinely used dialysis tubing is assumed to retain material with a molecular weight of 12,000 and higher. The dialysate was lyophilized to a dry powder which was redissolved to 25 ml (the original volume of total extract) and both this portion and the material retained by the dialysis sac were resterilized for testing. The same procedure was used for dialysis through cellophane tubing No. 23/32, which is impermeable to molecules of mol wt greater than 6,000 (9). Ultrafiltration of the dialysate was carried out with Diaflo UM-2 membranes (Amicon ultrafiltration cell model 202, Amicon, N.V., The Hague, The Netherlands). The dry powder obtained from 6.5 ml of dialysate was redissolved in 150 ml water and ultrafiltered at room temperature under 40 psi pressure, with water added intermittently until a total of 375 ml of filtrate was collected. This filtrate was lyophilized and redissolved in 6.5 ml water.

Preparation of Cell Suspensions.—Spleens were removed aseptically from 1–3 month old intact or thymectomized C57Bl/6 male or female mice and dispersed by pressure through a stainless steel mesh into organ culture medium (6) or Tyrode's solution. The cells were further dissociated by passage through a syringe with 26- or 27-gauge needles. Portions of cells were stained with Turk's solution and nucleated cells were counted in a hemacytometer. Control spleen cell suspensions were prepared in a similar manner from (C3H/eb x C57BL/6)F_1 mice.

Exposure of Spleen Cells to Extracts.—Exposure of spleen cells to extracts was carried out (*a*) by addition of extracts to the medium during the course of the 4 day graft-*versus*-host assay, (*b*) by incubation of spleen cell suspensions with extracts for 1 hr prior to assay of immunocompetence, (*c*) by incubation of spleen explants in thymus extracts for 18–24 hr, or (*d*) by intraperitoneal injection of extracts into parental animals. In experiments in which spleen cells from C57BL/6 thymectomized mice were exposed to extracts of thymus or spleen or fractions of these extracts during the course of the graft-*versus*-host assay, each extract (prepared as described above) was diluted 1:50 in culture medium and used in place of the regular nutrient medium surrounding each pair of cultures. In experiments in which the cells from C57BL/6 thymectomized mice were incubated with extracts prior to the graft-*versus*-host assay of immunological activity, 0.5 ml portions of dissociated spleen cells in culture medium were added to equal volumes of diluted extracts. Incubation of cells was

performed for 1 hr in a shaking water bath at 37°C. The cells were washed 2 times in culture medium, centrifuged for 3 min at 1500 rpm, resuspended, counted, and added to newborn spleen explants. When in vivo assay was performed, the cells were concentrated by centrifugation without washing, resuspended, counted, and injected to recipient hybrid animals. Incubation of spleen explants with thymus extract was accomplished by addition of extract diluted 1:50 in the culture medium in which explants of 2 × 1 × 1 mm were maintained. After the tissue had been incubated 18–24 hr in a 37°C incubator (under the same conditions to be described for the in vitro graft-*versus*-host assay) cell suspensions were prepared from the pooled explants. Injection of extracts of thymectomized donor mice consisted of 5 daily intraperitoneal injections of 0.5 ml double-strength extract (lyophilized powder dissolved in half the original volume). On the 6th day the spleens of these mice were tested for immunocompetence. Injection of extracts to normal mice consisted of one 1 ml intraperitoneal injection 2 hr or 18–24 hr before assay of their immunocompetence.

Test of Immunocompetence In Vitro.—Immunocompetence of spleen cells was evaluated by the ability of cell suspensions from C57BL/6 mice to induce enlargement of (C3H/eb x C57BL/6)F_1 newborn spleen explants, according to the method developed by Auerbach and Globerson (6). As described previously (5), cultures were considered reactive when the index of splenomegaly obtained was 1.2 or more.

Test of Immunocompetence In Vivo.—According to the method of Simonsen (7), 1×10^7 dissociated spleen cells from C57BL/6 mice were injected intraperitoneally into 8 day old (C3H/eb x C57BL/6)F_1 host mice. Each recipient litter included mice injected with each type of donor cell, as well as uninjected controls. The recipients were sacrificed 8 days after challenge and the ratio of mg spleen:10 g body weight was measured. Spleen index was obtained by dividing the spleen:body weights by the corresponding value of the uninjected control animals, since spleen weights of uninjected controls is equivalent to mice injected with syngeneic cells.

RESULTS

The ability of dissociated lymphoid cells to induce a graft-*versus*-host reaction against tissues of allogeneic origin is one important criterion of the immunological competence of these graft cells. Spleen cells from C57BL mice are known to induce a graft-*versus*-host response in tissues of (C3H × C57BL)F_1 hybrids both when injected in vivo and when tested in vitro against spleen explants from newborn F_1 mice, as indicated by a consequent reaction of splenomegaly on the part of the host (6, 7). Spleen cells from neonatally thymectomized mice fail to initiate such a process, indicating that immunological reactivity in this response is thymus dependent (5, 10). Using an in vitro assay of graft-*versus*-host reactivity we have shown that whereas spleen cell inocula prepared from neonatally thymectomized C57BL/6 mice did not induce enlargement of (C3H/eb × C57BL/6)F_1 newborn spleen explants, spleen explants challenged by portions of the same parental cells in the presence of syngeneic thymus extract consistently initiated a reaction of splenomegaly. Thus the dissociated spleen cells attained immunological reactivity by the direct action of a noncellular component of syngeneic thymus (5).

In Vitro Repair of Immunocompetence by Thymus Extracts of Allogeneic and Xenogeneic Origin.—The first experiments were performed to determine whether this activity of thymus extract is species-specific or whether, as sug-

gested by previous in vivo experiment, the competence-inducing action of a thymic factor can cross the barrier between strains and between species (4). Thus extracts of thymus or of spleen were prepared from mice of three strains allogeneic to the C57BL/6 spleen cells tested. These extracts were checked for the ability to restore graft-*versus*-host reactivity to spleen cells from neonatally thymectomized mice during the course of the 4 day assay. Thymus or spleen extract was added to the culture medium surrounding the (C3H/eb × C57BL/6)F_1 spleen fragments under challenge by 1×10^6 C57BL/6 spleen cells from thymectomized donors. Cells from the same donor were divided between

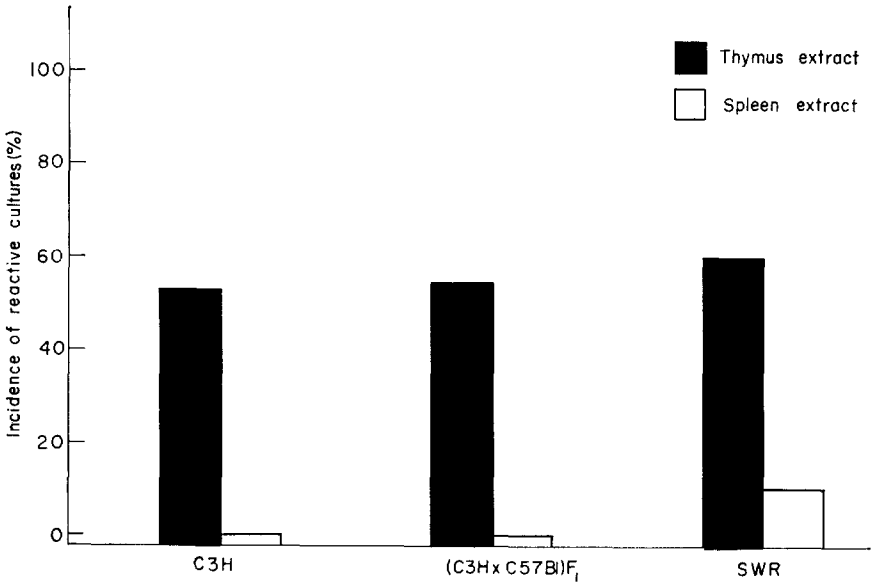

FIG. 1. In vitro graft-*versus*-host response induced by spleen cells from neonatally thymectomized C57BL/6 mice in the presence of allogeneic thymus or spleen extract. Dissociated spleen cells from thymectomized donors and (C3H/eb x C57BL/6)F_1 spleen explants were maintained throughout the course of the 4 day assay in culture medium supplemented with extracts from thymus or spleen. Cultures with spleen index $\geq$ 1.2 were considered reactive.

cultures maintained in the presence of thymus extract or of spleen extract and each test fragment was compared to its paired reference explant challenged by cells from intact (C3H/eb × C57BL/6)F_1 mice. Extract from each allogeneic strain (0.2 mg protein/ml) was tested in a total of 19–20 replicate cultures in three separate assays. As can be seen in Fig. 1, the capacity of spleen cells from thymectomized mice to induce splenomegaly was apparent when allogeneic thymic extract was present during the assay, while cells from the same thymectomized donors exposed to spleen extract of the same protein concentration, failed to induce significant splenomegaly. Thus thymus extract pre-

pared from C3H/eb, SWR/Jax, or (C3H/eb × C57BL/6)F_1 mice conferred to cells from thymectomized C57BL/6 mice the ability to react against F_1 tissues.

The results of similar experiments in which extracts prepared from calf thymus were tested in the 4 day graft-*versus*-host assay in vitro are presented in Table I. Spleen cells (1×10^6) from neonatally thymectomized C57Bl/6 donors attained the ability to induce a graft-*versus*-host reaction in the presence of calf thymus extract (0.2 mg protein/ml) in the culture medium surrounding each pair of explants while portions of the same cells in the presence of extract of similar protein concentration from calf spleen failed to react. As can be seen in Table I, when ultracentrifugation at 100,000 *g* was prolonged from 1 hr to 5 hr, activity of the supernatant was increased and reached a level previously obtained with extracts of syngeneic thymus (75% of cultures responding). Since it is accepted that particulate components of homogenized cells are precipitated after 5 hr centrifugation at 100,000 *g*, the active principle of the thymus extract would appear to be derived from a soluble component of thymus tissue.

TABLE I

In Vitro Graft-versus-Host Response Induced by Spleen Cells from Neonatally Thymectomized C57BL/6 Mice in the Presence of Calf Thymus or Spleen Extract

Extract tested*	Incidence of reactive cultures‡					Cultures responding
						%
Thymus ▲	3/5	0/5	2/5	4/10	3/6	39
Spleen ▲	0/5	1/5	0/5	0/10	0/6	3
Thymus ●	4/4	6/7	5/9	5/5	4/7	75
Spleen ●	0/5	1/7	0/9			5

* Extracts of calf thymus or spleen centrifuged at 100,000 *g* for 1 hr (▲) or 5 hr (●) were tested at a concentration of 0.2 mg protein/ml culture medium throughout the course of the 4 day assay.

‡ Number of cultures with a spleen index ≥ 1.2 per total number of cultures tested.

Separation of the Active Principle of Thymic Humoral Factor by Exhaustive Dialysis and Ultrafiltration.—Once it was demonstrated that thymic extracts of bovine origin can elicit graft-*versus*-host reactivity by direct activation of spleen cells from neonatally thymectomized mice, material of bovine origin was used as the source of thymic factor and separation of the active principle was attempted by means of exhaustive dialysis. Using cellophane dialysis casing No. 27/32, we dialyzed 25 ml portions of thymus extract against volumes of 500 ml 0.005 M phosphate buffer for 60 hr in the cold. The two portions obtained (the dialysate and the residue retained within the sac) were tested in the 4 day in vitro assay of graft-*versus*-host reactivity at concentrations corre-

sponding to the 0.2 mg protein/ml of complete thymus extracts described above. To achieve the desired concentration, the material retained by the dialysis sac was diluted 1:50 in culture medium after sterilization. The dialysate was dried by lyophilization, redissolved in 25 ml (the volume of the original total extract), sterilized and diluted 1:50 in the culture medium surrounding the spleen explants under test. Spleen cells (1×10^6) from neonatally thymectomized C57BL/6 donor mice were then tested for the ability to induce splenomegaly in the presence of each preparation. The results summarized in Table II indi-

TABLE II

In Vitro Graft-versus-Host Response Induced by Spleen Cells from Neonatally Thymectomized C57BL/6 Mice in the Presence of Dialyzed Extracts of Calf Thymus or Spleen

Extract tested*		Type of dialysis sac	Incidence of reactive cultures‡				Cultures responding
Organ	Fraction						
							%
Thymus ▲	Dialysate	No. 27/32§	3/4	5/5	5/8	2/5	68
Thymus ▲	Retained in dialysis sac	No. 27/32	1/5	1/5		0/5	13
Thymus ●	Dialysate	No. 27/32	3/5	3/5	5/10	5/7	59
Spleen ●	Dialysate	No. 27/32		1/4	0/10	0/6	5
Thymus ●	Dialysate	No. 23/32‖	5/7	6/7	2/3	3/4	76
—	—	—	0/5	0/5	0/5		0

* Fractions obtained after exhaustive dialysis from extracts of calf thymus or spleen centrifuged at 100,000 *g* for 1 hr (▲) or 5 hr (●). Fractions were tested at a 1:50 dilution in culture medium and were present throughout the course of the 4 day assay.

‡ Number of cultures with a spleen index ≥ 1.2 per total number of cultures tested.

§ Permeable to particles of molecular weight of 12,000 or less.

‖ Permeable to particles of molecular weight of 6000 or less.

cate that the active principle of thymus extract passes through a dialysis sac No. 27/32 during exhaustive dialysis, while the portion retained in the dialysis sac was inactive. The dialysate of spleen extract was also devoid of activity. When a similar exhaustive dialysis was performed with dialysis tubing No. 23/32 (impermeable to molecules of molecular weight greater than 6,000) the active fraction of the thymus extract was also recovered in the dialysate (Table II). Dialysate of calf thymus extract was then filtered through Diaflo membrane UM-2. When the filtrate obtained was lyophilized, redissolved to the original volume, and tested at a 1:50 dilution in the graft-*versus*-host assay, the competence-inducing activity of the filtrate was apparent (Fig. 2).

It was of interest to determine whether the dialysate of calf thymus extract

could activate spleen cells to immunocompetence after a short period of incubation prior to antigenic stimulation, as was seen previously with crude extracts of syngeneic thymus (5). For this purpose, spleen cells from neonatally thymectomized C57BL/6 mice were incubated in 1 ml culture medium containing dialysate of thymus extract (calf thymus extract centrifuged at 100,000 *g* for 5 hr) at the same concentration used in the previous experiment. After 1 hr incubation the cells were washed twice in 5 ml of culture medium and immunocompetence of an inoculum of 1×10^6 cells was evaluated by the in vitro assay of splenomegaly. The results presented in Table III indicate that activation

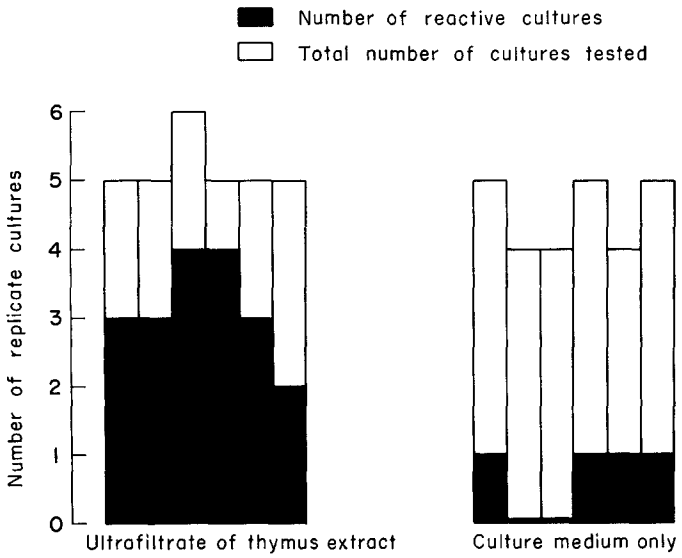

FIG. 2. In vitro graft-*versus*-host response induced by spleen cells from neonatally thymectomized C57BL/6 mice in the presence of ultrafiltrate of calf thymus extract.

of spleen cells from thymectomized donors was also achieved by 1 hr incubation with dialysate of thymus extract.

Restoration of Immunocompetence of Thymectomized Mice by Injections of Dialysate of Thymus Extract.—At this point in the investigation it seemed necessary to determine whether the dialysate of thymus extract which conferred immunological competence in vitro, could also restore the reactivity of spleen cells from thymectomized mice when injected into these animals. For this purpose, 6-week old neonatally thymectomized C57BL/6 mice were injected with 0.5 ml double strength dialysate of the thymus extract (see Materials and Methods). On the 6th day after 5 daily intraperitoneal injections, cell suspensions were prepared from the spleens of these animals and immunocom-

petence of 1×10^6 cells evaluated by graft-*versus*-host assay in vitro and in vivo.

The results obtained by in vitro assay are presented in Table IV where it can be seen that injections of dialysate of thymus extract restored the reactivity of spleen cells in 5/5 thymectomized mice after 5 days of treatment. Spleen cells from mice injected in parallel with an equivalent preparation of spleen

TABLE III

In Vitro Graft-versus-Host Response Induced by Spleen Cells from Neonatally Thymectomized C57BL/6 Mice Preincubated for 1 hr in Dialysate of Calf Thymus Extract

Incubation medium	Incidence of reactive cultures*				Cultures responding
					%
Dialysate of calf thymus extract‡	3/5	5/5	3/6	3/6	64
Culture medium	0/5	0/4	1/4	1/6	10

* Number of cultures with a spleen index ≥ 1.2 per total number of cultures tested.

‡ Obtained after exhaustive dialysis in cellophane dialysis tubing No. 27/32 from calf thymus extract and diluted 1:50 in culture medium.

TABLE IV

In Vitro Graft-versus-Host Response Induced by Spleen Cells from Neonatally Thymectomized C57BL/6 Mice Treated with 5 Daily Injections of Dialysates of Calf Thymus or Spleen Extracts

Extract injected*	Incidence of reactive cultures‡					
Thymus	4/5	3/6	3/5	4/4	4/5	
Spleen	1/4	1/5	2/5	0/5	0/4	0/4

* Five daily intraperitoneal injections of 0.5 ml of dialysates corresponding to 20 mg/ml protein prepared from extracts of calf thymus or spleen. Exhaustive dialysis performed with cellophane dialysis tubing No. 27/32.

‡ Number of cultures with a spleen index ≥ 1.2 per total number of cultures tested with cells from individual donor mice.

extract failed to induce significant splenomegaly. A further indication of restoration by means of in vivo administration of dialysate from thymic extract is presented in Fig. 3. Neonatally thymectomized C57BL/6 mice were injected daily with 0.5 ml double-strength dialysate of thymus extract and spleen cell suspensions prepared on the 6th day were tested in the Simonsen assay. Dissociated spleen cells (1×10^7) were injected into 8-day old (C3H $\times$ C57BL)F_1 hybrids and the index of splenomegaly was determined 8 days later. Again, restoration of immunocompetence of spleen cells from mice injected with calf thymus dialysate is apparent. The incidence of splenomegaly induced by these

cells from treated mice was significantly greater than that caused by control thymectomized donors. Most of the mice challenged with spleen cells from thymus dialysate injected donors manifested an index of splenomegaly greater than 1.4, while none of the mice injected with cells from control thymectomized donors showed a response above this index.

Since extracts of thymus tissue were shown to affect spleen cells from thymectomized mice by conferring reactivity on incompetent populations of spleen cells, it seemed of interest to determine whether these extracts have some measurable effect on populations of lymphoid cells from intact mice

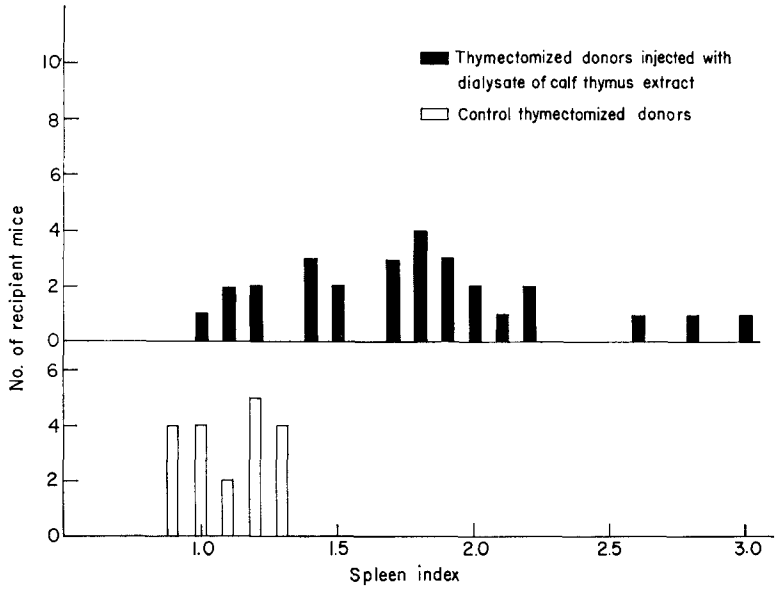

FIG. 3. In vivo graft-*versus*-host response induced in (C3H/eb x C57Bl/6)F_1 recipients by spleen cells from neonatally thymectomized C57Bl/6 mice injected on 5 successive days with dialysate of calf thymus extract.

(Table V). For this purpose, 1×10^5 spleen cells from normal mice were assayed for graft-*versus*-host reactivity since such inocula do not contain a sufficient number of competent cells to induce a graft-*versus*-host reaction, as can be seen in the table. When thymus extract was present during the course of the 4 day assay, the incidence of graft-*versus*-host response was only slightly higher (27% as compared to 14% in untreated controls). Also when explants of parental spleen tissue were incubated 18–24 hr previously with thymus extract, inocula of 1×10^5 cells prepared from the incubated tissue failed to induce a graft-*versus*-host response. However, when suspensions of 1×10^5 spleen cells were prepared from intact mice given a single injection of 1 ml of calf thymus

extract 18–24 hr previously, the presence of enough competent cells in the inocula was manifested by induction of a graft-*versus*-host response. These results suggested that the proportion of competent cells in the spleen was increased by the action of thymus extract in the intact mouse. Since 1×10^5

TABLE V

In Vitro Graft-versus-Host Response Induced by Spleen Cells from Intact C57BL/6 Mice Exposed to Calf Thymus Extract

Conditions of exposure	No. of cells tested	Incidence of reactive cultures							Response	
									Cultures responding	No. of donor mice responding
									%	
—	1×10^6	3/3	3/4	4/4	3/3	4/4	4/4		95	
—	1×10^5	0/4	1/6	1/5	1/5	0/4	1/5		14	
Calf thymus extract present during GvH assay in vitro	1×10^5	1/6	2/6	2/6	1/4				27	
Incubation of spleen explants with calf thymus extract 18–24 hr in vitro	1×10^5	0/5	0/9	0/6	0/5				0	
Injection of calf thymus extract 18–24 hr previously	1×10^5	4/6	4/7	5/7	3/4	3/4	2/5	3/5		6/7
Injection of syngeneic thymus extract 18–24 hr previously	1×10^5	4/4	2/5	3/6	4/7	4/6				4/5
Injection of calf thymus extract 2 hr previously	1×10^5	1/4	0/4	4/6 0/5	0/4 2/5	3/4	3/4			3/8

spleen cells were also active after injection of syngeneic thymus extract, this effect did not seem to be merely the result of recruitment of cells in response to an antigenic challenge. Injection of calf thymus preparation 2 hr before assay resulted in a less consistent effect, suggesting a gradual increase in the proportion of competent cells within the spleen under the influence of thymus factor acting on target cells originating outside the spleen (Table V).

DISCUSSION

The results of these experiments indicate that the thymic factor which confers competence upon immunologically defective populations of lymphoid cells in vitro is neither strain nor species specific and confirm the interspecies reactivity suggested previously by repeated injections of thymus preparations to thymectomized mice (4).

Isolation of the active factor from xenogeneic extracts was begun by high speed centrifugation for 5 hr. The resulting supernatant showed increased competence-inducing activity in comparison to preparations centrifuged for 1 hr. After exhaustive dialysis of calf thymus preparations for 60 hr through routinely used cellophane dialysis casing (No. 27/32) into a 20 times greater volume of dilute phosphate buffer, the active portion of the thymus extract was found in the dialysate. Moreover, this factor was recovered from the dialysate after a similar procedure was carried out with dialysis tubing (No. 23/32) of smaller porosity. By this criterion the dialyzable thymic agent would seem to consist of particles of molecular weight of 6000 or less. A further physical separation of the dialysate of thymic extract by ultrafiltration through a Diaflo UM-2 membrane indicated that the molecular weight of the active principle of thymic extract is of the order of magnitude of 1000 or less. These results are in apparent discrepancy with previous experiments performed in this laboratory in which the thymic humoral factor was characterized as a nondialyzable protein or protein-bound material on the basis of an assay of lymphocytopoietic activity (11). While the present characterization is based on exhaustive dialysis of thymic preparation the previous conclusion was derived largely from dialysis performed in equilibrium conditions. Moreover, an assay based on a measure of immunological competence rather than on lymphopoietic activity appears to us to be more relevant to the clarification of the mechanism of thymus functions. It is interesting to note at this point that activity of another thymic preparation shown to enhance thymidine incorporation in lymphoid cells was found to be associated with a slowly dialyzable protein fraction (12).

Use of the in vitro assay of graft-*versus*-host reactivity permitted us to isolate an active fraction of thymus extract which is both dialyzable and filterable. As a further evaluation of the conclusions indicated by the in vitro assay, spleen cells of neonatally thymectomized mice injected with a dialysate of thymus extract were also tested in the Simonsen assay of graft-*versus*-host activity in vivo. The results obtained after 5 daily injections of the dialysate of calf thymus extract to neonatally thymectomized animals confirmed the restoration of immunocompetence indicated by assay in vitro.

Once it was demonstrated that a humoral factor of thymic tissue could confer immunological reactivity upon spleen cell populations with impaired competence resulting from neonatal thymectomy, a logical step was to test the effect of thymic humoral factor on populations of spleen cells of intact mice.

In order to detect a measurable increase of immune reactivity in intact mice, we tested inocula of 1×10^5 spleen cells which were found previously to contain insufficient numbers of competent cells to induce a graft-*versus*-host response in vitro (5). Thymus extracts did not modify the competence of these populations to an extent detectable by initiation of a response when added in vitro either to suspensions of cells or to fragments of spleen tissue from which cell suspensions were subsequently tested. However, when thymus extract was injected to intact mice and similar cell inocula tested 18–24 hr later, the capacity to initiate a graft-*versus*-host response was apparent. These results, obtained after injection of either bovine or syngeneic preparations, seemed to be the expression of a gradual process which was not completed 2 hr after injection. This increase in the number of competent cells within the spleen population may be explained by an inflow of cells and suggests the existence of a source of incompetent cells which may be activated by a thymus factor in normal mice.

SUMMARY

By means of an assay of graft-*versus*-host activity some properties of the thymic humoral factor which confers immunocompetence upon lymphoid cells in vitro have been studied. Allogeneic and xenogeneic thymic preparations were found to activate lymphoid cells from neonatally thymectomized mice, enabling initiation of a graft-*versus*-host response. Thus, this thymus factor is apparently neither strain nor species specific.

The active principle of calf thymus extracts was found to be in the supernatant after prolonged ultracentrifugation. When exhaustive dialysis and ultrafiltration through Diaflo membranes were performed, the active thymus agent was found to pass through both the dialysis sac and Diaflo UM-2 membranes. The molecule which confers immunocompetence upon lymphoid cells thus seems to be of molecular weight of an order of magnitude of 1000 or less. Dialyzed thymus preparations injected into neonatally thymectomized mice also restored the capacity of spleen cells of these mice to induce graft-*versus*-host activity. When injected into intact mice, thymus extract also increased the proportion of competent cells in the spleens of these animals, probably by activation of target cells originating outside the spleen.

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