IN VITRO STUDIES ON STRAIN-DEPENDENT PRODUCTION OF THYMUS-SPECIFIC AUTOANTIBODIES*

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In previous studies in this laboratory, a plaque assay was developed in which cells secreting antibodies lytic for nucleated cells could be enumerated as plaque-forming cells (PFC)¹ (1-3). This procedure has been successfully employed in studies on the appearance, in the spleens of stimulated mice, of PFC producing antibodies to xenogenic (1) and allogenic (2, 3) cell-surface antigens and in studies on the genetic control of the magnitude of antibody response to certain alloantigens (4, 5).

More recently these studies have been extended to in vitro experiments in which normal mouse spleen cells were stimulated in cultures by allogenic thymus cells (6). Besides the expected PFC response to θ -AKR antigen of stimulating cells, we observed the appearance of small numbers of PFC that lysed thymus cells of all murine strains tested, including syngenic thymus cells. This finding of in vitro formation of what appeared to be thymus-specific autoantibodies stimulated the present investigation.

The major objective of this study was to find out the conditions under which the number of autoantibody-secreting PFC can be increased by in vitro stimulation of spleen cells with syngenic and autologous thymus cells or their products. In addition to thymus cells, murine lymphoma L5178Y cells were used as a target for the detection of PFC and of antibodies released to the cell culture medium. Most experiments dealt with stimulation of spleen cells from strain DBA/2J, which is syngenic with L5178Y lymphoma. However, other murine strains were also included in these studies for the purpose of comparison. Finally, in vitro formation of thymus-specific autoantibodies was compared with in vivo formation of such antibodies by DBA/2J mice stimulated with syngenic thymus cells.

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¹ *Abbreviations used in this paper:* ME, 2-mercaptoethanol; PBS, phosphate-buffered saline; PFC, plaque-forming cells; TL, thymic leukemia antigen.

Materials and Methods

Animals.—Female mice of inbred strains DBA/2J (DBA/2), AKR/J (AKR), NZB/BINJ (NZB), and C57BL/6J (C57BL/6), 8–10 wk old, were purchased from the Jackson Laboratory, Bar Harbor, Maine. (DBA/2 $\times$ NZB) F_1 hybrids were bred in the animal facility of this university. Fetuses of DBA/2 mice were obtained at 16–19 days of gestation. Gestational stages were estimated by counting the appearance of vaginal plugs as day 0.

Cell Cultures.—Cell suspensions from spleen or thymus removed from an individual mouse or pooled from three to eight normal mice were prepared aseptically in cold balanced salt solution, pH 7.2, by pressing the minced tissues through stainless steel wire screen, 50 mesh, and then filtering through 200-mesh screen. The resulting cell suspensions were centrifuged at 200 g for 10 min at 4°C and resuspended in an appropriate volume of the culture medium. The cell concentration of the suspensions was determined by counting cells stained with crystal violet; and the cultures were prepared in plastic Petri dishes, 35 mm in diameter, by the technique of Mishell and Dutton (7), with slight modifications. RPMI 1640 medium was used rather than Eagle's medium; it was supplemented with 5% fetal calf serum (Rehatain, Reheis Chemical Co., Chicago, Ill.). Each culture consisted of 1 ml of a suspension containing 2×10^7 spleen cells or 2×10^7 thymus cells or a mixture of 10^7 spleen cells and 10^7 thymus cells. In some experiments, cells of an individual surgically removed spleen were cultured. L5178Y lymphoma cells were also cultured in a few experiments, 1 ml of a suspension containing 10^6 cells being used. The cultures were maintained in an atmosphere of 10% CO₂, 7% O₂, and 83% N₂ and "fed" daily with 0.1 ml of nutritional mixture (7). Supernatants of cultures were separated from cells by centrifugation at 200 g for 10 min at 4°C and stored at -70°C until tested for cytolytic antibodies.

To test the supernatants of thymus cell cultures for their activity to stimulate spleen cell cultures, the supernatants were made cell free by filtration through Nuclepore membranes (General Electric Co., Pleasanton, Calif.), pore size 0.5 μ m, and used immediately or after storing at -70°C. To 0.5 ml of spleen cell cultures containing 2×10^7 cells, 0.5 ml of the cell-free supernatants were added at time 0.

Murine Lymphoma Cells.—DBA/2 lymphoma cell line L5178Y, which was established and cloned by Fischer (8), and C57BL/6 lymphoma, EL4, were maintained in syngenic mice as an ascites tumor by weekly transfer of 0.1 ml of ascitic fluid. Ascites cells were harvested 6–7 days after transplantation of the tumor.

PFC Assay.—Cultured cells harvested from three dishes were pooled. After centrifugation at 200 g for 10 min, cells were resuspended in 0.9 ml of M199 and were assayed for PFC by the previously described procedure, but with slight modifications (2, 3). Lymphoma L5178Y or thymus cells were employed as target cells. To 0.3 ml of 0.7% Difco-Bacto agar (Difco Laboratories, Detroit, Mich.) dissolved in M199 and containing 0.5 mg DEAE-dextran/ml were added, at 45°C, 0.05 ml of the target cell suspension (5×10^6 L5178Y or 2×10^7 thymus cells) and 0.05–0.1 ml of cultured cell suspension. The mixture was spread on a microscopic slide precoated with a 0.1% agar. Slides were placed in a humidified chamber and incubated for 1 h at 37°C in an atmosphere of 5% CO₂ in air. Fresh rabbit serum diluted 1:10 in M199 (complement) was added, and the slides were kept for $\frac{1}{2}$ h at 4°C and for 1 h at 37°C. Thereafter slides were fan-dried, fixed in 95% ethanol for $\frac{1}{2}$ h, rinsed in tap water, and dried again. Plaques were counted on unstained slides under strong, indirect illumination. Each sample was assayed in triplicate, and the number of PFC recovered per dish (culture) was calculated.

The assay for PFC producing antibodies to sheep red cells (SRC) was done in a similar manner except that 0.05 ml of a 5% suspension of SRC were added per slide as target and fresh guinea pig serum diluted 1:10 was used as a source of complement.

Titration of Cytolytic Antibodies by Spot Tests.—Supernatants of cultures were usually concentrated about five times by means of osmotic dialysis against hypertonic sucrose solution,

which was followed by dialysis against phosphate-buffered saline (PBS), pH 7.2. If not studied immediately, supernatants were preserved at -70°C .

Titration of antibodies by cytotoxicity in agar, by spot tests, was carried out by the previously described procedure, with slight modifications (2). The agar layer containing proper target cells was prepared as in the PFC assay except that target cells alone were added to the agar. $5\ \mu\text{l}$ of twofold serial dilutions of supernatants were placed on the surface of an agar layer containing proper target cells, and the preparations were left in a humid chamber at 4°C for 1 h. Slides were washed for $\frac{1}{2}$ h in cold Hanks' solution and incubated at 37°C for 1 h with rabbit serum diluted 1:10 in M199 (complement), or M199 alone as control. Thereafter, slides were processed as in the PFC assay. Cytotoxicity could be recognized as a circular refractile spot under indirect illumination. The reciprocal of the highest dilution of the supernatant at which partial but still definite cytotoxicity was observed was taken as the endpoint titer. Each titration was done in duplicate.

Absorption of Antibodies.—Absorption of antibodies was performed by adding graded numbers of lymphoid cells to 0.2 ml of the supernatant and mixing them continuously on an electric rotator for 1–2 h at 4°C . In the case of absorptions with liver and kidney cells, the cells were mechanically dispersed in cold M199 from the tissues of normal mice in a manner similar to that described for lymphoid cells. They were washed three times in cold M199 and sedimented by centrifugation at $8,000\ g$ for 10 min. 0.2 ml of packed cells were mixed with an equal volume of the supernatant at 4°C and processed in a manner similar to that described for lymphoid cells. Supernatants were separated by centrifugation at $8,000\ g$ for 10 min. Up to three absorptions were conducted. For control absorptions, suspensions of cells heated at 100°C for 15 min were used. In some experiments, absorption was carried out at 22°C and at 37°C as well as at 4°C .

Physicochemical Characterization of Antibodies.—Gel filtration was carried out by using a Sephadex G-200 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), $30\ \text{cm} \times 1.5\ \text{cm}$, equilibrated with 0.01 M PBS, pH 7.2. 1 ml of a concentrated culture supernatant was placed on the column, and 2-ml fractions were collected. Sucrose density gradient centrifugation was done by layering 0.5 ml of a concentrated supernatant on 4.0 ml of a preformed linear gradient of sucrose ranging from 0.3 to 0.7 M and centrifuging at $130,000\ g$ for 18 h. Fractions were dialysed against PBS at 4°C for 18 h before titration for cytotoxic antibodies.

Mercaptoethanol Treatment.—Equal volumes of a supernatant and 0.1 M 2-mercaptoethanol (ME) were mixed and incubated for 1 h at 37°C . Titration was done after dialysing ME-treated samples for 18 h against PBS at 4°C .

In Vivo Stimulation with Syngenic Thymus Cells.—Normal DBA/2 mice were injected intravenously with 0.1 ml of a cell suspension containing 8×10^7 syngenic thymus cells, prepared in cold M199 in a manner similar to that described above. Heparin at a dose equivalent to 10 USP units was given intraperitoneally to each animal before the injection of thymus cells. Uninjected mice served as controls.

RESULTS

In Vitro Development of PFC Detectable on Syngenic Thymus and Lymphoma Cell Targets.—Lymphoid cells from normal individual DBA/2 mice were cultured as described under Materials and Methods. Three groups of cultures—spleen cells alone (S), thymus cells alone (T), and a mixture of equal numbers of spleen and thymus cells (S+T)—were prepared with a total cell inoculum of 2×10^7 cells per culture. The cultured cells were examined by plaque assay with DBA/2 thymus or L5178Y lymphoma cells as targets (Table I). Virtually no PFC were detected at time 0. In the course of cultures, S developed a small

TABLE I
PFC in Cultures of S, T, or S + T. Assays on Autologous and Syngenic Thymus Cells and L5178Y Lymphoma Cells

Animal no.	Cells in culture	PFC/culture, day 4, assayed on	
		DBA/2 thymus	L5178Y
1	S	5	18
	S + T*	11	498
	T	0	0
2	S	12	7
	S + T*	4	90
	T	0	0
3	S	6 (6)	9
	S + T	13 (12)	278
4	S	16 (15)	20
	S + T	18 (31)	184
5	S	n.t.	24
	S + T*	n.t.	572
	S + T	n.t.	670
	T	n.t.	0
6	S	n.t.	95
	S + T*	n.t.	892
	S + T	n.t.	832
	T	n.t.	0
7	S	n.t.	30
	S + T*	n.t.	514
	S + T	n.t.	594
	T	n.t.	0

S and T from individual DBA/2 mice were cultured alone with an initial cell inoculum of 2×10^7 cells per culture. S + T* was an autologous cell mixture of 10^7 S and 10^7 T. S + T indicates a similar mixture except that T was pooled from three to five syngenic mice. The numbers in parentheses are the number of PFC detected on thymus target cells autologous with S. n.t., not tested.

number of PFC that released antibodies lytic to syngenic thymus cells, scoring an average of 10 PFC per culture on day 4. In S+T cultures, a number of PFC similar to that in S cultures was demonstrated on a syngenic thymus target. In some experiments, autologous thymus cells were used in parallel to syngenic thymus cells as a target for PFC. Comparable numbers of PFC were detected on autologous and syngenic targets. Assays of S cultures on L5178Y lymphoma cells as target showed PFC in numbers similar to those noted in assays on syngenic thymus target. In S+T cultures, on the other hand, a markedly

increased number of PFC was demonstrated on L5178Y lymphoma cells. The numbers scored on day 4 varied from 90 to 892 PFC per culture among seven individual animals tested. S+T cultures composed of autologous and of syngenic cells showed comparable numbers of PFC detectable on an L5178Y target. No PFC detectable on either thymus or L5178Y targets were noted in T cultures.

Fig. 1 illustrates the time course of the appearance of PFC detectable on L5178Y cells. In S+T cultures, 410 PFC per culture were scored on day 4, 27

FIG. 1. The appearance in lymphoid cell cultures of DBA/2 mice of PFC detectable on L5178Y lymphoma cells. Spleen cells (S) and thymus cells (T) were pooled from eight normal DBA/2 mice and cultured alone with an initial cell inoculum of 2×10^7 cells per culture. S + T was a mixture of 10^7 S and 10^7 T. At each time point the cultured cells harvested from three dishes were pooled and assayed for PFC with L5178Y cells as the target.

times more than in S cultures. The PFC number declined below 100 PFC per culture on days 5 and 6.

In order to clarify whether the presence of thymus cells themselves is required for the enhancement of the PFC response directed to L5178Y cells in S+T cultures, we have examined the effects of cell-free supernatants of T cultures on such responses. As shown in Table II, the enhanced PFC response to L5178Y could be induced by adding cell-free supernatants of 24–96-h cultures of T to S cultures at time 0. The supernatants of the T cultures of 24 h already showed maximal activity.

Similar results were obtained with cell-free supernatants of L5178Y cultures whereas those of S cultures were without effect. The observed enhancement did not seem to be due to any nonspecific enhancing factor(s) because anti-SRC PFC that appeared without addition of SRC in S cultures were not affected by the addition of T or L5178Y supernatants.

Cytolytic Antibodies Appearing in the Culture Supernatants.—Culture supernatants were tested for antibodies lytic to syngenic thymus and L5178Y cells by means of spot tests. Hardly any lysis of these target cells was induced by

TABLE II
Effect of Cell-Free Supernatants of the Cultures of T, S, and L5178Y on the PFC Response to L5178Y

Experiment no.	Cells in cultures	Cell-free supernatants added	PFC/culture, day 4, assayed on	
			L5178Y	SRC
1	S	—	8	108
	S	T, 24 h*	298	99
	S	T, 48 h*	330	93
	S	T, 72 h*	264	84
	S	T, 96 h*	264	93
	S + T	—	242	66
2	S	—	14	453
	S	T, 48 h*	404	267
	S	S, 48 h*	18	272
	S	L5178Y, 48 h*	840	171
	S + T	—	482	327

Supernatants of DBA/2 T or S cultures, 2×10^7 cells per culture, or of L5178Y, 10^6 cells per culture, were made cell free as described under Materials and Methods. To 0.5 ml of DBA/2 S cultures containing 2×10^7 cells, 0.5 ml of the cell-free supernatants were added at time 0. As a control, 0.5 ml of the fresh culture medium replaced the supernatants. S + T was a mixture of 10^7 DBA/2 S and 10^7 DBA/2 T.

* The length of the time for which T, S, or L5178Y cells were cultured before the supernatants were separated from the cells.

any supernatants except those of S+T cultures, in which antibodies lytic to L5178Y cells could be shown with an average titer of 1:16 on day 5 (Fig. 2). Physicochemical analysis of such antibodies in the concentrated supernatants of S+T cultures was carried out by means of gel filtration on Sephadex G-200 and sucrose density gradient centrifugation (Fig. 3). Antibodies lytic to L5178Y cells were demonstrated in the first peak of Sephadex fractions and in the bottom fractions of the sucrose density gradient. Reductions of the antibodies by ME completely abolished their cytolytic activity. These results indicated that the antibodies under study were of the IgM class.

Absorption of Antibodies Lytic to L5178Y Cells with Cells from Normal Syngenic Mice.—In order to find out whether the antibodies lytic to L5178Y cells

FIG. 2. The appearance of antibodies lytic to L5178Y lymphoma cells in the supernatants of lymphoid cell cultures of DBA/2 mice. Supernatants of the cultures from the experiments shown in Fig. 1 were separated from cells by centrifugation at 200 g for 10 min and stored at -70°C until tested. Titration of cytolytic antibodies in unconcentrated supernatants was done by spot tests with L5178Y cells as the target.

that appeared in S+T cultures were directed to antigens present on normal syngenic cells or to antigens expressed only on L5178Y cells, absorption of the supernatants of S+T cultures by cells from normal syngenic mice was carried out. As shown in Table III, antibodies lytic to L5178Y cells could be completely absorbed from concentrated supernatants of S+T cultures by thymus cells of normal DBA/2 mice or by L5178Y cells. Similar results were obtained when cells of a surgically removed spleen were cultured with syngenic thymus cells and the culture supernatants were absorbed with thymus cells autologous with the spleen cells. Thymus cells or L5178Y cells heated at 100°C for 15 min did not absorb antibodies.

The absorption experiments also demonstrated that removal of antibodies was more efficient at 4°C than at 22°C . At 37°C hardly any absorption took place (Fig. 4). Absorption with spleen, lymph node and bone marrow cells as well as with erythrocytes at numbers comparable with those of thymus cells failed to affect antibodies. Multiple absorptions with packed cells of liver and kidney also failed to cause any significant reduction of the titer of supernatants, and the results obtained with fresh and heated cells were similar.

The antigen thus demonstrated in normal thymus was not restricted to the DBA/2 strain. Absorptions of the supernatants with thymus cells of normal AKR and C57BL/6 mice removed antibodies as effectively as absorptions with DBA/2 thymus cells (Table III). On the other hand, C57BL/6 lymphoma, EL4, did not absorb antibodies.

FIG. 3. (a) Gel filtration of supernatant of S + T cultures of DBA/2 mice on Sephadex G-200 column, 30 cm $\times$ 1.5 cm, equilibrated with 0.01 M PBS, pH 7.2. 1 ml of a concentrated supernatant was placed on the column and 2-ml fractions were collected. (b) Sucrose density gradient centrifugation of supernatant of S + T cultures of DBA/2 mice. 0.5 ml of a concentrated supernatant was layered on 4.0 ml of a preformed linear gradient of sucrose ranging from 0.3 to 0.7 M and centrifuged at 130,000 g for 18 h. Fractions were dialyzed against PBS for 18 h before titration for antibodies. Titration of cytolytic antibodies in each fraction was done by spot tests with L5178Y cells as the target.

TABLE III
Absorption of Antibodies to L5178Y in the Concentrated Supernatants of S + T

Strains of cell donors	Cells used for absorption	Number of absorptions	Titer of antibodies to L5178Y in the supernatants of S + T		
			Absorbed with		Unabsorbed
			Fresh cells	Heated cells*	
DBA/2	Thymus, 2×10^8 cells	1	2	128	256
DBA/2	Thymus, fetal,† 5×10^7 cells	1	<2	128	256
DBA/2	L5178Y, 5×10^7 cells	1	<2	128	128
DBA/2	Spleen, 2×10^8 cells	1	64	64	256
DBA/2	Lymph node, 2×10^8 cells	1	64	64	256
DBA/2	Bone marrow, 2×10^8 cells	1	64	64	256
DBA/2	Erythrocytes, 2×10^8 cells	1	64	64	256
DBA/2	Liver, 0.2 ml of packed cells	1	32	32	128
DBA/2	Liver, 0.2 ml of packed cells	3	32	32	128
DBA/2	Kidney, 0.2 ml of packed cells	1	64	64	128
DBA/2	Kidney, 0.2 ml of packed cells	3	32	32	128
AKR	Thymus, 2×10^8 cells	1	<2	128	256
C57BL/6	Thymus, 2×10^8 cells	1	<2	128	256
C57BL/6	EL4, 2×10^8 cells	1	64	64	128

The supernatants of S + T cultures of DBA/2 mice were osmotically concentrated about five times. Absorption was carried out by adding cells to 0.2 ml of the supernatants and mixing them continually for 2 h at 4°C or, in the case of multiple absorptions, 1 h for each absorption. Titration of cytolytic antibodies was done by spot tests with L5178Y cells as the target.

* Cells were heated at 100°C for 15 min.

† Fetuses at day 16–19 of gestation.

The results described above provided evidence that the antibody response to L5178Y that occurred in S+T cultures of DBA/2 mice was directed to antigens present on thymus cells of all murine strains tested, including syngenic and autologous thymus cells.

Fig. 5 illustrates the quantitative absorption of antibodies with graded numbers of normal syngenic thymus and L5178Y cells. It was shown in this experiment that the absorbing efficiency of L5178Y cells was 30 times greater than that of normal thymus cells, indicating that the antigen under discussion may either be more strongly expressed on L5178Y than on thymus cells or be expressed only on a minor population of thymus cells.

Demonstration of the Antigen on Fetal Thymus.—Since the results suggested that normal adult DBA/2 spleen cells could recognize an antigen present in the animal's own thymus and respond to it in vitro with the formation of antibodies, experiments were carried out to determine whether this antigen is present during fetal life. Thymus cells from 16–19-day-old fetuses of DBA/2 mice were pooled and used for absorption of supernatants of S+T cultures. Antibodies lytic to L5178Y cells could be completely removed by fetal thymus

FIG. 4. Absorption at different temperatures of cytolytic antibodies from the supernatants of S + T cultures of DBA/2 mice. To 0.2-ml samples of a concentrated supernatant, graded numbers of L5178Y cells were added. The mixtures were kept continuously rotated for 2 h at different temperatures as indicated. Titration of cytolytic antibodies remaining in the supernatants was done by spot tests with L5178Y cells as the target.

cells (Table III). It was also demonstrated that the supernatants of 24–48-h cultures of T from 16-day-old fetuses stimulated PFC responses to L5178Y cells in adult S cultures as effectively as similar preparations from adult thymus cells (Table IV).

PFC Response Against L5178Y in S+T Cultures from Strains Other than DBA/2.—As shown above, the thymic antigen under study was not restricted to the DBA/2 strain, but could be demonstrated in all other strains tested. We next studied whether spleen cells of strains other than DBA/2 respond to this antigen upon stimulation in vitro with syngenic or allogenic thymus cells. Experiments performed with syngenic S+T cultures of strains C57BL/6 and NZB as well as (DBA/2 $\times$ NZB) F_1 hybrids (Table V) did not show any significant increase in the number of PFC detectable on L5178Y when compared with S. Hardly any increase of the PFC number was demonstrated in S cultures of NZB and (DBA/2 $\times$ NZB) F_1 origin by the addition of T from allogenic mice. In contrast, S cultures from DBA/2 showed the expected increase in the number of PFC detectable on L5178Y upon addition of T from NZB or (DBA/2 $\times$ NZB) F_1 as well as from DBA/2.

FIG. 5. To 0.2-ml samples of a concentrated supernatant of S + T cultures of DBA/2 mice, graded numbers of DBA/2 thymus cells and L5178Y cells were added. The mixtures were rotated continuously for 2 h at 4°C. Titration of cytolytic antibodies remaining in the supernatants was done by spot tests with L5178Y cells as the target.

TABLE IV
Effects of Cell-Free Supernatants of Fetal and Adult T Cultures

Cells in culture	Cell-free supernatants of T cultures	PFC/culture, day 4, assayed on L5178Y
S	—	78
S	Fetal, 24 h*	672
S	Fetal, 48 h*	736
S	Adult, 24 h*	454
S	Adult, 48 h*	358
S + T	—	244

Supernatants of fetal or adult DBA/2 T cultures, 2×10^7 cells per culture, were made cell free as described under Materials and Methods. Fetal T cells were obtained at day 16 of gestation. To 0.5 ml of adult DBA/2 S cultures (2×10^7 cells per culture) were added 0.5 ml of the cell-free supernatants at time 0. As a control, 0.5 ml of the fresh medium replaced the supernatants. S + T was a mixture of 10^7 S and 10^7 T of adult DBA/2 mice.

* The length of the time for which T cells were cultured before supernatants were separated from the cells.

TABLE V
Effects of the Strain on the PFC Response to L5178Y Cells Occurring in the Cultures of S and S + T

Cells in culture		PFC/culture, day 4, assayed on L5178Y cells
S	T	
C57BL/6	—	51
C57BL/6	C57BL/6	33
NZB	—	32
NZB	NZB	70
NZB	DBA/2	48
NZB	(DBA/2 × NZB)F ₁	94
(DBA/2 × NZB)F ₁	—	22
(DBA/2 × NZB)F ₁	NZB	50
(DBA/2 × NZB)F ₁	DBA/2	48
(DBA/2 × NZB)F ₁	(DBA/2 × NZB)F ₁	50
DBA/2	—	10
DBA/2	NZB	360
DBA/2	DBA/2	424
DBA/2	(DBA/2 × NZB)F ₁	326

S and T cells pooled from three or four mice of each strain were employed for these experiments. The initial cell inoculum of S culture was 2×10^7 cells per culture. S + T was a mixture of 10^7 S and 10^7 T.

In Vivo Stimulation with Syngenic Thymus Cells.—Finally, DBA/2 mice were injected intravenously with syngenic thymus cell suspensions to see whether spleen cells of such mice could respond also in vivo to the antigen under study. Development in the spleen of PFC detectable on L5178Y was demonstrated on day 4 after the intravenous injection of 8×10^7 syngenic thymus cells in all of 10 animals. PFC ranged from 130 to 3,060 per spleen, the geometric mean number being 741. The appearance of serum antibody lytic to L5178Y cells could also be shown in these animals. Among 10 uninjected control animals, 8 had fewer than 20 PFC in their spleens, and 2 animals had 38 and 83 PFC per spleen, respectively.

DISCUSSION

The results of this study have shown that spleen cells of normal DBA/2 mice have the capacity to form in vitro antibodies directed to certain antigen(s) present on the animal's own thymus as well as to syngenic lymphoma L5178Y cells. By means of plaque assay we found that S cultures developed a small number of PFC detectable on both syngenic thymus and L5178Y cells, whereas S+T cultures developed a markedly increased number of PFC that were

detectable on L5178Y cells, but not on thymus cells. It seems, therefore, that two populations of antibody-forming cells participated in the observed reactions.

One population of PFC was detectable in S cultures by the lysis of syngenic thymus cells and possibly also L5178Y cells. This population did not seem to be affected by adding T to S cultures. The spontaneous occurrence in S cultures of the PFC that released antibodies lytic to syngenic thymus cells has already been demonstrated in a previous *in vitro* study (6), and seems to be a general phenomenon occurring in spleen cell cultures of any mouse strain. These *in vitro* results are consistent with the *in vivo* observations that mice of various strains have in their sera the natural autoantibodies cytotoxic to thymus cells (9, 10). In our *in vitro* experiments, however, such antibodies were also produced by the spleen cells of all the mice, which were only 8 wk old (younger animals were not tested), whereas mice studied for serum antibodies by other investigators were positive after 5 mo of age. The significance of such autoantibody formation remains unclarified.

The other population of PFC detectable on L5178Y cells but not on thymus cells was demonstrated in S+T cultures only, though a small number of PFC of this population might possibly be present in S cultures. They appeared in the cultures apparently as a result of the stimulation of S with certain immunogenic substances released by T. This assumption is supported by the fact that cell-free supernatants of T cultures or of L5178Y cultures added to the syngenic S cultures induced a specific increase in the number of PFC detectable on L5178Y.

The obvious explanation for the stimulation of syngenic and even autologous S by the substances released from T would be that the immune response is directed against antigens altered or acquired during the course of cultures. This, however, did not appear plausible since freshly prepared syngenic thymus cells possessed such antigen(s), shown by the absorption of the antibodies lytic for L5178Y. Thus we are led to conclude that cells capable of recognizing surface antigens present on the animal's own thymus cells exist in the spleens of normal DBA/2 mice.

The antigen under study had two outstanding characteristics: (a) it could be demonstrated on thymus cells, but not on any other murine tissue cells; and (b) its presence on thymus cells was not restricted to DBA/2, but was demonstrable in all other murine strains tested. Although the tissue distribution of the antigen resembled that of the thymic leukemia (TL) antigen (11), it was shown that the antibodies lytic to L5178Y cells could also be absorbed by thymus cells from TL-negative strains such as AKR and C57BL/6.

The results of quantitative absorption experiments indicated that L5178Y cells were 30 times more efficient in removing the antibodies than were thymus cells. This result is consistent with the fact that antibodies lytic for L5178Y cells failed to lyse thymus cells, and may indicate that the antigen is either more strongly expressed on L5178Y lymphoma cells than on thymus cells or is expressed only in a minor population of thymus cells. The latter possibility is

supported by the fact that the θ -positive lymphoma EL4 of C57BL/6 origin (12) did not absorb the antibodies whereas the comparable number of C57BL/6 thymus cells absorbed them completely, which may indicate heterogeneity in thymus cells with regard to the presence of the antigen under study. L5178Y lymphoma cells, which in our hands are also θ positive, may thus have been derived from the minor population of thymus cells carrying the particular antigen.

Physicochemical characterization of the antibodies in S+T cultures showed that they were of IgM class. It was further demonstrated that the binding of the antibodies to the antigen was more efficient at 4°C than at 22°C and that hardly any binding occurred at 37°C. It is of interest to note that natural thymocytotoxic autoantibodies demonstrated in the sera of NZB mice by Shirai and Mellors (10) were also IgM antibodies with optimal activity at 4°C and were directed against an antigen present on thymus cells of all strains tested. However, there are distinct differences between the antibodies lytic to L5178Y described here and those found in NZB mice: (a) NZB autoantibodies are thymocytotoxic (10) and thymocytolytic as tested by cytolysis in agar,² whereas the antibodies under discussion are not; and (b) the antigen reacting with NZB antibodies is detected on cells from thymus, spleen, lymph node, and brain, whereas the antigen of this study seems to be restricted to thymus.

It has recently been reported that in vitro autosensitization of normal lymphoid cells of rats and mice as measured by cell-mediated immunity could be induced against antigens on syngenic fibroblasts (13) and syngenic thymus reticulum cells (14). The authors suggested the existence of lymphocytes that are reversibly tolerant to autoantigens and of regulatory mechanisms that may function in vivo to prevent differentiation of self-tolerant lymphocytes. The stimulation observed in our in vitro studies apparently does not occur, or seldom occurs, in vivo under physiologic conditions. The vast majority of normal DBA/2 mice examined in this study did not have any PFC detectable on L5178Y cells in their spleens. On the other hand, an intravenous administration of fresh syngenic thymus cells has readily stimulated such animals, and the PFC lytic for L5178Y cells could be detected in the spleens 4 days after the injection. It would appear, therefore, that the thymus cells carrying the stimulating antigen do not enter the circulation under physiologic conditions and, consequently, no PFC response occurs in the spleen of normal mice.

Another possible explanation of the recognition by DBA/2 mice of the antigens of the animal's own thymus would be that the antigen appears in the thymus after self-tolerance is established. Although our demonstration of the antigen in the fetal thymus of 16 days' gestation argues against this explanation, it cannot be excluded entirely.

Significantly, our experiments showed that the ability to respond to the self

² Auer, I., T. Tomasi, and F. Milgrom. 1973. Natural thymocytolytic autoantibodies in NZB and other strains of mice. *Cell. Immunol.* In press.

antigen under study is a strain-dependent phenomenon. Spleen cells of NZB and C57BL/6 mice did not respond to such antigen in vitro whereas DBA/2 spleen cells have readily responded to stimulation in vitro and even in vivo. It is thus possible that the lack of self-tolerance observed in DBA/2 mice is due to genetically determined effects. It was shown that spleen cells of (DBA/2 $\times$ NZB) F_1 hybrids behaved like NZB cells, which suggests that the ability to respond to the thymic autoantigen may be a recessive trait.

The strain NZB is well known for spontaneously developing autoantibodies to various antigens (15), and genetic studies (16-20) have shown that one or more genes may be responsible for this property. The observation that NZB mice were negative for the autoantibody response in this study indicates that a different genetic control is involved here. To our knowledge there have not been any other reports on autoantibody production by DBA/2 mice, and the immunopathological significance of the phenomenon described in this study has to be clarified in the future. Moreover, the present in vitro system may provide a good means of studying the cellular basis of generations of self-tolerance and of its breakdown.

Finally, it may be postulated that the demonstrated antigen is of extrinsic origin like the antigens associated with infection by vertically transmitted leukemia viruses. Although such a possibility cannot be completely excluded, we have no data to substantiate it. Our results only stress the possible genetic effects on the ability to respond to cell-surface antigens expressed on the animal's own cells.

SUMMARY

In vitro cultures of spleen cells (S) from normal 8-10-wk-old DBA/2J mice were shown to develop a small number of plaque-forming cells (PFC) that released antibodies lytic to syngenic and autologous thymus cells as well as to syngenic lymphoma L5178Y cells used as the target in the PFC assay. A marked increase in the number of PFC detectable on L5178Y target cells was demonstrated on day 4 in the cultures of S cells to which syngenic or autologous thymus cells had been added (S+T) at time 0, whereas the PFC detectable on thymus cells in such cultures remained at a level similar to that in S cultures. This suggested that two populations of PFC participated in the observed phenomena. No PFC developed in the culture of thymus cells (T). The addition of the cell-free supernatants of 24-h cultures of T or of L5178Y cells to syngenic S cultures also caused a specific increase in the number of the PFC detectable on L5178Y, which suggested that certain immunogenic factors released from the T cells stimulated the response observed in the S+T cultures.

Antibodies of IgM nature were detected in the supernatants of S+T cultures by means of cytolysis in agar of L5178Y cells. Although such antibodies did not cause lysis of thymus cells, they could be completely removed by absorption with normal adult or fetal thymus cells of syngenic origin. Still, the absorbing

capacity of L5178Y was much higher than that of thymus cells. The absorption was more efficient at 4°C than at 22°C, and hardly any absorption occurred at 37°C. The tissue distribution of the antigen under study seemed to be restricted to thymus cells since no other murine tissue cells tested removed the antibodies.

The thymic antigen under study was not restricted to strain DBA/2J and could be demonstrated on thymus cells of all other strains tested. On the other hand, the ability of spleen cells to respond *in vitro* to this antigen has thus far been observed only in DBA/2J mice. Spleen cells of strains C57BL/6J and NZB/BINJ as well as (DBA/2 × NZB)F₁ failed to show any significant increase in the PFC response detectable on the L5178Y target when syngenic thymus cells or DBA/2J thymus cells were added.

An intravenous injection of syngenic thymus cells to DBA/2J mice also caused the appearance in their spleens of PFC detectable on the L5178Y target.

The described *in vitro* system may provide a good means of studying the cellular basis of generation of self-tolerance and of its breakdown.

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