AUTOSENSITIZATION IN VITRO*

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Sensitized lymphocytes, rather than circulating antibodies, appear to mediate autoimmune damage to solid organs. This has been demonstrated in experimental autoimmune diseases involving various organs such as brain (1, 2), thyroid (3–6), or kidney (7) in laboratory animals. The role of cellular immunity in most human autoimmune diseases is less clearly defined (8), although cell-mediated reactions have been demonstrated in conditions such as ulcerative colitis (9).

The origin of lymphocytes capable of reacting with self-antigens and the conditions in which these lymphocytes are induced to react are not well understood. A basic question is whether or not potentially self-reactive lymphocytes exist in the naturally self-tolerant adult animal. According to the clonal selection theory as originally proposed by Burnet (10, 11), natural tolerance to self-antigens results from the ontogenic elimination of potentially self-reactive lymphocytes. An alternative possibility is that potentially self-reactive lymphocytes are not eliminated by contact with self-antigens, but persist in adult animals as unresponsive or tolerant cells.

The occurrence of autoimmune diseases can be explained by activation of potentially self-reactive cells as well as by circumvention of an elimination mechanism. According to Burnet (10), autoimmunity may result from the interaction of mature lymphocytes with self-antigens which were "inaccessible" during development. In addition, self-reactive cells may arise by mutation of lymphocytes after the critical period of elimination.

Since these possibilities are difficult to evaluate in intact animals, the question of the origin of self-reactive lymphocytes has not been resolved in studies of experimentally induced autoimmune states.

In this paper we present evidence which suggests that lymphocytes potentially reactive with accessible self-antigens may indeed exist in mature animals. We used a closed system of primary sensitization in which lymphocytes capable of mediating cellular immune reactions are selected and sensitized in vitro (12–17). By this method it was possible to demonstrate that mature rats or

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mice carry lymphoid cells which can be autosensitized against syngeneic fibroblasts.

Materials and Methods

Animals.—Inbred mouse strains C3H/ebJ (H-2^k) or C57BL/6J (H-2^b) were obtained from Jackson Laboratories, Bar Harbor, Maine. Lewis rats (AgB-1) were supplied by Microbiological Associates, Inc., Bethesda, Md. A strain of Wistar rats (AgB-unknown) in its 15th generation of brother-sister inbreeding was obtained from Mr. Joseph Shalom of the Department of Biodynamics of this Institute.

The purity of the mouse and Lewis rat strains was confirmed by skin transplantation. The histocompatibility of the Wistar strain was tested and found complete by transplantation of various organs and by parabiosis experiments. In addition, three monthly intraperitoneal injections of 10^8 pooled Wistar spleen cells in complete Freund adjuvant failed to elicit cytotoxic antibodies in 25 Wistar rats against Wistar leukocytes.

Autosensitization In Vitro.—Only male animals were used as donors of lymph node or spleen cells when they were about 3 months of age. Suspensions of lymphoid cells were prepared in cold phosphate-buffered saline (PBS)[1] by using a fine wire mesh as described previously (15). The cells were counted, centrifuged, and resuspended in Dulbecco's modification of Eagle's medium (EM) which contained 20% horse serum (for rat cell culture) or 20% fetal calf serum (for mouse cells). About 35×10^6 cells, in 4 ml of medium, were seeded on each sensitizing fibroblast monolayer. Fibroblast monolayers were prepared and maintained as described previously (12–17). Sensitizing monolayers contained 3×10^6 fibroblasts in 60 mm diameter plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.). In some experiments 1 μg/ml of hydrocortisone sodium succinate or prednisolone sodium tetrahydrophthalate was added to cell cultures to promote sensitization (12, 15). On the 3rd day of culture, 3 ml of medium was replaced by fresh EM containing added serum but not glucocorticoids. The lymphoid cells were collected from the sensitizing fibroblast monolayers after 5 days of culture by repeated pipetting of the medium. The cells were washed in cold PBS, counted, and assayed for their ability to mediate immune effects. The number of lymphoid cells recovered after sensitization was about 20–30% of the number originally sown in the absence of added glucocorticoids, and about 1–5% in cultures which contained glucocorticoids during sensitization.

Assay of Cytolytic Effects.—Target fibroblast cultures contained 0.7×10^6 fibroblasts in 35 mm plastic Petri dishes. The target fibroblasts were labeled with ^{51}Cr by incubating the cultures for 24 hr with medium containing 2 μCi/ml as described previously (12, 15). Sensitized lymphoid cells in 1.5 ml of medium were incubated with target fibroblasts for 20 hr. The per cent of lysis of target fibroblasts was measured as the per cent of ^{51}Cr label released into the medium, minus that measured in control cultures (12–15).

Assay of Graft vs. Host Reactions.—Sensitized lymphoid cells in 0.1 ml of PBS were injected intraperitoneally into newborn rat or mouse littermates within 24 hr of birth. The animals were weighed daily. Significance of differences in weight (runting) was determined by the Student *t* test. Spleen indices were computed by the formula of Simonsen (18).

RESULTS

Cytolysis of Syngeneic Fibroblasts In Vitro by Autosensitized Rat Lymphocytes.—It was found in previous studies that rat lymph node cells could be

[1] *Abbreviations used in this paper*: EM, Eagle's medium; GvH, graft vs. host; PBS, phosphate-buffered saline.

sensitized in vitro against foreign rat or mouse fibroblasts (12, 13, 16, 17). These lymph node cells were found to transform into large lymphocytes, which were able to lyse target fibroblasts in vitro. Table I shows the results of two experiments in which Lewis or Wistar rat lymph node cells were sensitized against allogeneic or xenogeneic (mouse) fibroblasts. These experiments demonstrate the immunospecificity of the lytic phase of the in vitro reaction (13, 16). Although unrelated target fibroblasts are usually lysed to a slight degree, the amount of lysis of target fibroblasts of the sensitizing genotype is always several times greater (13).

Autosensitization against syngeneic fibroblasts was attempted using the same in vitro system. Table II shows the results of an experiment in which Wistar lymph node cells were sensitized in vitro against Wistar fibroblasts.

TABLE I

Sensitization and Cytolytic Effects of Rat Lymph Node Cells against Foreign Fibroblasts

Sensitization phase*			Lytic phase‡	
Rat lymph node cells	Sensitizing fibroblasts	Large lymphocytes	Target fibroblasts	Lysis
		(%)		(%)
Lewis	C3H	51	C3H	23 ± 0.3
			Lewis	3 ± 0.6
Wistar	Lewis	45	Lewis	21 ± 1.1
			C3H	3 ± 0.2

* 20×10^6 Lewis rat lymph node cells were sensitized in vitro.

‡ 2×10^6 sensitized lymph node cells were incubated with each target culture.

Autosensitization was done with or without added prednisolone, because it was found that glucocorticoids in the presence of antigens acted to select specifically sensitized lymphocytes in vitro (12). After the sensitization phase of the experiment, 23% of lymphocytes recovered from cultures without prednisolone and 54% of lymphocytes recovered from cultures with prednisolone appeared to be large transformed cells. Cytolytic effects of the autosensitized lymphocytes were measured against syngeneic Wistar rat or C3H mouse target fibroblasts. It was found that lysis of the syngeneic Wistar fibroblasts was considerably greater than that of C3H fibroblasts.

As shown in Table II, populations of lymphocytes sensitized in the presence of prednisolone differed from those sensitized without prednisolone in that they did not decrease in cell number during the lytic phase, were relatively richer in large cells, and produced more lysis of target fibroblasts. Although the number of lymphocytes recoverable after lysis appeared to be similar in target cultures of either genotype, lysis of syngeneic Wistar target fibroblasts was 10–20 times greater than that of mouse fibroblasts. Thus, syngeneic fibroblast antigens

appeared to activate the cytolytic mechanism of autosensitized lymphocytes to a degree of immunospecificity comparable to that achieved by in vitro sensitization against foreign fibroblasts (Table I and reference 13). Table III shows the results of three experiments in which cytolysis of Lewis or C3H target cells was mediated by autosensitized Lewis lymph node cells. Immunospecific lysis of syngeneic target fibroblasts was demonstrated in each of these experiments.

GvH Reactions in Newborn Rats Produced by Autosensitized Cells.—It is possible that the sensitization and cytolytic reactions demonstrated above were induced by, and directed against, "foreign" antigens present on the syngeneic fibroblasts. Culturing fibroblasts in vitro may have modified their surface

TABLE II

Autosensitization and Cytolytic Effects of Wistar Rat Lymph Node Cells

Sensitization phase*			Lytic phase				
			No. of lymphocytes × 10^6				
Prednisolone	Large lymphocytes	Target fibroblasts	Prelysis		Postlysis		Lysis
			Total	Large	Total	Large	
(μg/ml)	(%)						(%)
0	23	Wistar	1.6	0.4	0.9	0.1	21 ± 0.7
		C3H	1.6	0.4	1.1	0.3	1 ± 0.4
1	54	Wistar	1.6	0.9	1.6	1.0	48 ± 2.4
		C3H	1.6	0.9	2.0	1.1	5 ± 0.4

* 18 × 10^6 Wistar lymphocytes were sensitized against syngeneic fibroblasts with or without prednisolone added to the medium.

antigens, or exposed "inaccessible" or "embryonic" antigens which are not available on fibroblasts found in the intact animal. If this were the case, lymphocytes sensitized against syngeneic fibroblasts in cell culture might not be able to recognize and interact with unmodified self-antigens. It was necessary, therefore, to test whether or not these lymphocytes could react against self-antigens in vivo.

It has been shown that grafted lymphoid cells reacting against host-tissue antigens can produce a decrease in body weight (runting) or splenomegaly in newborn animals (18). The production of these GvH reactions by lymphoid cells sensitized in vitro served as a test of their ability to react against unmodified antigens in vivo.

Preliminary experiments were done in order to gauge the effects of injecting newborn rats with lymphoid cells which were sensitized in vitro against foreign antigens. Table IV shows the spleen indices in two litters of newborn Wistar rats which were injected with 5 × 10^6 Lewis rat spleen cells sensitized in vitro against C3H mouse or Wistar rat fibroblasts. There were no significant differ-

ences in the body weights of littermates. The spleen indices of Wistar rats injected with Lewis spleen cells sensitized against C3H antigens were no greater than 1.02. On the other hand, injection of Lewis anti-Wistar spleen cells produced spleen indices of 1.20–1.98. These findings indicate that the injection of spleen cells sensitized against unrelated antigens does not produce splenomegaly, and that spleen indices above 1.20 may be associated with an immunospecific reaction of the grafted spleen cells against the host.

TABLE III

*Cytolytic Effects of Autosensitized Lewis Rat Lymph Node Cells**

% Lysis of target fibroblasts‡	
Lewis	C3H
21 ± 1.1	3 ± 0.2
27 ± 0.4	5 ± 0.7
16 ± 0.9	4 ± 0.6

* 20×10^6 Lewis lymph node cells were sensitized in vitro.

‡ 3×10^6 sensitized cells were incubated with each target culture.

TABLE IV

*GvH Reaction in Rats Injected with Allosensitized Spleen Cells**

Litter No.	Spleen indices of newborn Wistar rats examined on day 11			
	Cells injected (5×10^6)			
	None	No. of rats	Lewis anti-C3H	Lewis anti-Wistar
1	1.00	3	0.98	1.98
			1.02	1.63
2	1.00	2	1.01	1.26
			1.02	1.20
			0.87	

* Lewis rat spleen cells (30×10^6) were sensitized against C3H or Wistar fibroblasts in the presence of hydrocortisone; 5×10^6 sensitized spleen cells (78% large cells) or PBS alone were injected into each newborn rat.

Table V presents the results of five separate experiments in which newborn Wistar rat littermates were injected with Wistar lymphoid cells sensitized in vitro against syngeneic fibroblasts. Control littermates were injected with PBS, with unsensitized cells, or with cells sensitized against C3H mouse fibroblasts. None of the control rats were runted and their spleen indices were not greater than 1.09. There were varied responses among the rats injected with autosensitized spleen cells. Litter No. 1 demonstrated the most striking reaction. Four of the five experimental animals had spleen indices of 1.49 or greater, and the fifth rat was markedly runted. Two of the four experimental rats in

litters 2 and 3 had spleen indices of 1.28 and 1.39. There were no spleen indices above 1.20 in litters 4 and 5, but three rats were runted.

These findings indicate that newborn rats may develop relatively enlarged spleens or runting, after the injection of lymph node or spleen cells sensitized in vitro against syngeneic fibroblasts. This suggests that autosensitized lymphocytes can recognize and interact with unmodified self-antigens present in vivo.

GvH Reactions in Newborn Mice Produced by Autosensitized Cells.—We recently developed a method for sensitizing mouse spleen cells against allogeneic

TABLE V

GvH Reaction in Wistar Rats Injected with Autosensitized Lymphoid Cells

Litter No.	Days after injection	Cells injected		Control rats		Spleen index	
		No. ($\times 10^6$)	Type	Injected cells	No. of rats	Experimental	Control
1	17	9.4	Lymph node	None	3	1.66	1.00
						1.49	
						1.73	
						2.12	
						1.01*	
2	8	20	Lymph node	Unsensitized	2	1.28	1.00
				Wistar cells		1.16	
3	14	20	Lymph node	Unsensitized	3	1.39	1.00
				Wistar cells		1.01	
4	6	5	Spleen‡	None	3	1.19*	1.00
				Wistar anti-C3H		1.02*	1.03
							1.01
5	11	5	Spleen‡	None	4	1.20*	1.00
				Wistar anti-C3H		1.10	1.09

* Significant ($P < 0.01$) reduction in body weight compared to control littermates.

‡ Hydrocortisone, 1 μg/ml, added to sensitization culture.

fibroblast antigens in cell culture (15). Allosensitized mouse spleen cells were able to produce immune effects in vivo such as GvH reactions (14) or rejection of tumor allografts (15). We therefore attempted to induce autosensitization of mouse spleen cells against syngeneic fibroblasts by using the same in vitro system. Functional autosensitization was assayed by studying the ability of these mouse cells to produce GvH reactions in syngeneic newborn mice.

Table VI shows the results of five different experiments in which C3H spleen cells were sensitized against syngeneic C3H, allogeneic C57BL mouse, or Lewis rat fibroblasts in vitro. The sensitized cells were injected into litters of newborn C3H mice, and body weight and spleen indices were measured. Simonsen (18) has reported that spleen indices above 1.30 after the injection of 10×10^6 cells can be considered as evidence of a significant GvH reaction. It was found that none of the mice injected with control spleen cells had a spleen

index above 1.16. The responses of the experimental mice varied considerably. However, at least one of the mice in each of the litters had a spleen index of 1.33 or greater. In litter No. 5 the three mice which were injected with 1×10^6 autosensitized spleen cells were all runted and had spleen indices of 1.57 or higher. On the other hand, a significant spleen index (1.48) was found only in one of two experimental mice injected with 10×10^6 spleen cells in litter No. 4.

This variation in the magnitude of the GvH reactions may be the result of variations in the degree of autosensitization achieved in different cultures and/

TABLE VI

*GvH Reaction in C3H Newborn Mice Injected with Autosensitized Spleen Cells**

Litter No.	Day post-injection	No. of spleen cells injected ($\times 10^6$)	Spleen cells injected as control	Spleen index‡	
				Experimental	Control
1	14	5	C3H anti-C57BL	1.42	1.16
				1.20	1.05
2	14	1	Unsensitized C3H	1.33	1.14
				1.12	1.15
				0.98	
3	7	5	C3H anti-Lewis	1.33	1.08
				1.35	1.14
4	7	10	—	1.48	
				1.17	—
5	12	1	Unsensitized C3H	1.66§	
				1.57§	1.15
				1.65§	

* Autosensitization was carried out in the presence of hydrocortisone.

‡ Compared to two or three littermates which were not injected.

§ Significant ($P < 0.01$) reduction in body weight.

or reflect differences between individual litters. Nevertheless, the findings suggest that mouse lymphoid cells, like those of the rat, may be sensitized against syngeneic antigens in vitro and that these cells are able to interact with self-antigens in vivo.

DISCUSSION

In these studies autosensitization appeared to be achieved in vitro in the two species in which it was attempted. Autosensitized rat cells were able to cause significant lysis of syngeneic rat, but not of mouse target fibroblasts (Tables II and III), and to produce splenomegaly or runting when injected into syngeneic newborn rats (Table V). Autosensitized mouse cells were found to cause similar GvH reactions in vivo (Table VI).

It was shown previously (15) that mouse spleen cells allosensitized in vitro could inhibit the growth of tumor allografts in irradiated recipients, but were

incapable of causing lysis of target fibroblasts in vitro. In preliminary experiments we found that autosensitized mouse cells were also unable to lyse target cells. However, these spleen cells were found to mediate an immunospecific enhancement of the growth of syngeneic tumors in irradiated recipients (14).

The results of these studies may be related to problems concerning the cellular basis of natural self-tolerance. It is not likely that self-reactive lymphocytes arose by random mutation in the cell-culture system. The in vitro system seems to promote the proliferation only of predetermined lymphocytes which can interact with antigens on the sensitizing fibroblast. The presence of glucocorticoid hormones during sensitization appears to accentuate this process of selection (12). Hence, potentially, self-reactive lymphocytes probably had to exist in the donor animals for autosensitization to occur in vitro.

It is also difficult to argue that the sensitizing antigens on syngeneic fibroblasts were either inaccessible or foreign to lymphocytes in the intact animal. The ability of the sensitized lymphocytes to produce GvH reactions in vivo indicates that these cells both had access to their complementary antigens and receptors capable of interacting with these self-antigens. The unsensitized lymphocytes which initiated sensitization in vitro should have also been able to interact with self-antigens in vivo if the active autosensitized cells were their clonal descendants (10).

On the other hand, recent evidence suggests that cell-mediated immune reactions, like antibody production (19), may require the cooperation of at least two lymphoid cell types (20). Thus, the lymphoid cell which initiates the sensitized state may be triggered by an immunogen which differs from the antigenic structure which activates the immune effector cell. It is conceivable that culturing fibroblasts in vitro modifies their antigens so that they become immunogenic without changing their antigenicity or losing their cross-reactivity with self-antigens in vivo. In this way changes in the structure of self-antigens in vitro may render them immunogenic to antigen-sensitive cells. This process may lead to the immune differentiation of effector cells so that they can react against previously nonimmunogenic self-antigens in vivo.

Although this interpretation is compatible with our findings, it is challenged by the results of studies on the antigenic specificity of cell-mediated delayed hypersensitivity reactions. These investigations were carried out both in vivo (21) and in vitro (22). It was found that the same immunochemical structures which are required to induce primary sensitization are also necessary to provoke previously sensitized lymphocytes to mediate delayed-hypersensitivity reactions. Antigens which were not immunogenic would not stimulate sensitized cells to respond, even though they could readily react with preformed antibody (21). These findings suggest that effector cells are activated only by complete immunogens. Hence, the development of GvH reactions suggests that the same immunogenic self-antigens present on the autosensitizing fibroblasts in vitro

were also available in vivo. The nature of the reactive antigens was not identified in the present studies. They probably were not major histocompatibility antigens as evidenced by the relatively weak GvH reactions which were produced in vivo (18).

The ability of lymphoid cells to interact with self-antigens in vitro also has been demonstrated in several other systems. Micklem et al. reported (23) that lymphocytes in mature mice have receptors which are able specifically to bind syngeneic or autochthonous erythrocytes to form clusters or rosettes. Falk et al. (24) found that rat thymocytes incubated with various antigens in vitro secrete a factor which inhibits the migration of macrophages or spleen cells. Incubation with antigens derived from the rats' own tissues also led to secretion of the inhibitory factor. More recent studies by Falk and associates[2] have suggested that self-reactive lymphoid cells originating from the thymus may be generally unable to become effector cells. Our finding (14) that autosensitized mouse cells specifically enhance rather than inhibit syngeneic tumors may support this concept. However, the GvH reactions and the cytolytic ability of autosensitized rat lymphoid cells suggest that self-reactive cells are capable of generating at least some immune effects.

Immune reactivity against self-antigens also was demonstrated in the studies of Boyse, Lance, and their associates (25, 26), using a system of irradiation chimeras. They prepared stable C57BL chimeras which were populated with A-strain mouse lymphoid cells. However, A-strain skin grafts were rejected by chimeric mice which contained only A-strain lymphocytes in their circulation. The authors suggested that A-strain lymphocytes lost their natural tolerance to A-strain skin antigens after being transferred to C57BL recipients which lacked the specific skin alloantigen allele (25, 26). The mechanism by which self-tolerance was lost could not be analyzed in these in vivo studies.

The findings presented here indicate that ontogenic elimination of potentially self-reactive cells may not be the only basis for natural tolerance to self-antigens. Prevention of autoimmunity might depend, therefore, upon the existence of regulatory mechanisms which function in vivo to inhibit immune differentiation and replication of self-tolerant lymphocytes. An excess of antigen has been shown to induce tolerance to foreign tissues (27). Antigen excess or other regulatory mechanisms may also be important in maintaining self-tolerance in the intact animal. These regulatory mechanisms appear to be annulled in the cell-culture system leading to immune differentiation of previously self-tolerant lymphocytes. Thus, potential autosensitization can be realized in vitro. It is also possible that a decrease in the concentration of a specific skin antigen may have triggered the immune differentiation of potentially self-reactive cells in the C57BL–A-strain chimera mice in the system of Boyse and Lance (25, 26).

[2] Falk, R. E. Personal communication.

Preliminary evidence indicates that tolerance to allogeneic transplantation antigens induced in newborn mice can also be overcome in our cell-culture system. Loss of tolerance in the absence of mutation in vitro, therefore, can be explained most simply by the existence of lymphocytes which are reversibly tolerant to potential immunogens. Recovery from acquired tolerance to exogenous antigens is regularly observed to occur spontaneously, or can be induced by a variety of means (28). It has been suggested that reversibly tolerant cells indeed are the agents of acquired tolerance and its breakdown (29–31). Hence, it is conceivable that tolerant cells may form the basis of both acquired and natural tolerance.

SUMMARY

Autosensitization of rat or mouse lymphoid cells against syngeneic fibroblast antigens was induced in cell culture. Rat lymphoid cells autosensitized by this method were able to produce immunospecific lysis of syngeneic target fibroblasts in vitro or GvH reactions in newborn rats. Autosensitized mouse spleen cells mediated similar GvH reactions when injected into newborn mice.

The nature of the system used to induce immunity in vitro appears to argue against the possibility that lymphocytes capable of reacting against self-antigens could arise by mutation in cell culture. Hence, it is likely that cells potentially reactive against self-antigens preexisted in the lymphoid cell donors. The ability of autosensitized cells to mediate immune reactions in vivo suggests that the immunogenic self-antigens present on sensitizing fibroblasts also were accessible in the intact animals.

Loss of natural self-tolerance in vitro, therefore, can be explained most simply by the existence of lymphocytes which are reversibly tolerant to self. Hence, ontogenic elimination of potentially self-reactive cells may not be the only basis for natural tolerance. Regulatory mechanisms, such as antigen excess, may have to function in vivo to prevent differentiation of self-tolerant lymphocytes. These regulatory mechanisms appear to be annulled in the cell-culture system. The present system thus may offer a new approach to studies of tolerance and regulation of cellular immunity.

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