REGULATION OF AUTOSENSITIZATION

The Immune Activation and Specific Inhibition of Self-Recognizing Thymus-Derived Lymphocytes*

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Natural tolerance to self-antigens is a hallmark of the immune system. The mechanism by which self-tolerance is maintained may shed light upon the pathogenesis of autoimmune diseases as well as on the development of the immune system itself. Thus, the question of the cellular basis of self-tolerance has both practical and theoretical importance.

The nonreactivity of the immune system to self-antigens could be attributed to either of two basic mechanisms. Potentially self-reactive lymphocytes might be eliminated, or such lymphocytes might exist in a state of tolerance. The concept that potentially self-reactive lymphocytes are eliminated during development has been clearly outlined by Burnet. According to the elimination theory, autoimmune diseases occur because of the appearance of "forbidden clones" of immunocompetent lymphocytes that can recognize self-antigens. These forbidden clones can arise by somatic mutation of lymphocytes, or by contact of lymphocytes with self-antigens which were not accessible to the immune system during its development (1). Jerne has used the concept of lymphocyte elimination in an attempt to explain the generation of lymphocyte diversity in central lymphoid organs such as the thymus (2).

The hypothesis of lymphocyte elimination was tested experimentally in recent studies of autosensitization (3–5). The results of these studies indicated that self-tolerant lymphocytes do exist. We found that lymph node or spleen cells obtained from inbred rats or mice can be sensitized in vitro against syngeneic fibroblasts or thymus reticulum cells. Immunospecific cell-mediated damage to syngeneic target cells, or graft-*versus*-host (GvH)[1] reactions in vivo, served as a measure of thymus-derived (T) lymphocyte autosensitization. The nature of the in vitro system suggested that somatic mutation could not account for the development of forbidden clones. Furthermore, the thymus reticulum cells appear to be accessible in vivo to the T lymphocytes which

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[1] *Abbreviations used in this paper*: B cell, bone marrow-derived cell; EM, Eagle's medium; GvH, graft-*versus*-host; HS, horse serum; PBS, phosphate-buffered saline; T cell, thymus-derived cell.

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mediated autosensitization in vitro. It was therefore concluded that control mechanisms, rather than elimination of T lymphocytes, were responsible for self-tolerance in vivo. In this paper we describe the results of studies which confirm the existence of self-tolerant T lymphocytes, and demonstrate factors in autologous serum which specifically appear to inhibit autosensitization of these lymphocytes.

Materials and Methods

Animals.—Inbred Lewis and BN rats 6–8 wk old, and C3H/eb and BALB/c mice were obtained from the Animal Breeding Center of The Weizmann Institute of Science. Homozygosity was tested routinely by skin grafting. Cell or serum donors were anesthetized by using ether.

Fibroblast Cultures.—Monolayer cultures of rat or mouse fibroblasts were prepared from 16–17-day embryos as described previously (6–9). Secondary fibroblast cultures used for sensitization or as immunoadsorbents contained 2×10^6 cells in 60-mm-diameter plastic Petri dishes (Falcon Plastics, Oxnard, Calif.). Target fibroblast cultures contained 0.5×10^6 cells in 35-mm plastic Petri dishes. To prevent cell replication, the fibroblasts were irradiated with 2000 R from a cobalt-60 gamma source at a distance of 21.5 cm and a dose rate of 800 or 1000 R/min (150 A, Atomic Energy of Canada, Ltd., Ottawa). Target cultures were labeled with ^{51}Cr as described previously (8, 9).

Thymus Reticulum Cell Cultures.—Cultures of reticulum cells were prepared free of lymphocytes from rat thymus glands as described (4, 5). The reticulum cells were identified by positive periodic acid-Schiff staining and by their characteristic appearance in electron micrographs (H. Wekerle and I. R. Cohen, manuscript in preparation). Adsorbing or sensitizing monolayers contained 1.2×10^6 cells, and target monolayers contained 0.3×10^6 cells. These monolayers were not irradiated before use.

Autochthonous Thymus Sensitization Cultures.—Thymus glands from Lewis or BN rats were cultured individually to induce sensitization of thymus lymphocytes against autochthonous reticulum cells. The thymus glands were trimmed of extrathymic tissues. Cell suspensions were made by pressing each gland separately through a fine wire mesh into cold phosphate-buffered saline (PBS), pH 7.2. The cells of each thymus gland were washed by centrifugation (700 *g*, 6 min) and resuspended in 20 ml Dulbecco's modification of Eagle's medium (EM) containing 15% horse serum (HS) (Grand Island Biological Co., Grand Island, N. Y.); 10 ml of each cell suspension was cultured in 100-mm plastic Petri dishes (37°C, 10% CO_2 in moist air). The reticulum cells adhered to the Petri dish by the 3rd day of culture (4, 5). After 3 days, 6 ml of culture medium was removed and replaced by fresh EM + HS. The lymphocytes were separated from the reticulum cells after 5 days of culture by repeatedly washing the Petri dish with the culture medium. The lymphocytes were washed in PBS, counted in a hemacytometer, and resuspended in PBS in a concentration of 50×10^6 cells/ml. Autosensitization of the thymus cells was tested by the popliteal lymph node assay.

Lymphocyte Sensitization Cultures.—Lymphocyte suspensions were obtained from rat lymph node or thymus glands and cultured with syngeneic sensitizing monolayers. In kinetic experiments, in which the cells were cultured in vitro for 24 h or less, the sensitization cultures were incubated in EM without added serum. In these studies 28×10^6 lymphocytes in 4 ml EM were incubated in each sensitization culture. The lymphocytes together with the fibroblasts were collected from each plate using a rubber policeman, centrifuged, and resuspended in 0.4 ml of PBS; 0.2 ml was injected into the footpads of syngeneic rats to test autosensitization by the popliteal lymph node assay.

In other experiments, lymph node cells were sensitized against fibroblast monolayers in EM + HS for 5 days using a method previously described (3). Autosensitization was tested

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in these studies by transferring the lymphocytes to target fibroblast cultures and measuring their cytotoxic effects.

Assay of Cytotoxicity.—Lymphocytes from sensitization cultures or from popliteal lymph nodes were suspended in EM + HS and 1.5 ml were incubated with target fibroblast monolayers for 40–65 h. Percent lysis of target fibroblasts was measured in triplicate cultures as percent of ^{51}Cr label released into the culture medium, minus that measured in control cultures that did not contain sensitized lymphocytes (3, 7–9). In some experiments the percent of maximum lysis was computed by dividing the percent lysis in experimental cultures by the percent of ^{51}Cr released in control target cultures in which all the fibroblasts were lysed by quick freezing (liquid air) and thawing (37°C) in distilled water.

Popliteal Lymph Node Assay.—GvH reactions were measured by injecting 10×10^6 autosensitized lymphocytes in 0.2 ml PBS into the right footpads of groups of four syngeneic rats. Unsensitized lymphocytes were injected into the left footpads as controls. The popliteal lymph nodes were removed 6 days later and the cells of the right and left nodes of each group of rats were pooled and counted. An increase in the cell number of the right nodes compared with that of the left nodes served as a measure of the increase in lymph node size produced by a GvH reaction (10). A twofold or greater increase in cell number was considered as indicating a positive reaction (I. R. Cohen, manuscript in preparation). The immunospecificity of the reaction was tested by measuring the percent lysis of target fibroblast cultures produced by suspensions of lymphocytes from these lymph nodes.

Adherence of Lymphocytes to Adsorbing Fibroblasts.—Specific recognition of syngeneic fibroblast or reticulum cell antigens by normal lymph node cells was tested by a method previously used to detect lymphocyte receptors for foreign fibroblast antigens (11, 12). 2 ml of EM + HS containing 40×10^6 unsensitized lymph node cells were incubated with adsorbing monolayers of syngeneic cells for 4 h. 2 ml of fresh EM + HS were then added to the adsorption cultures and the nonadhering lymphocytes were separated from the adhering lymphocytes by repeated pipetting of the culture medium. The adherent lymphocytes were sensitized against the adsorbing monolayer cells by adding 4 ml of EM + HS and culturing the cells. The nonadherent lymphocytes were transferred to syngeneic or to foreign monolayers for sensitization. The degree of sensitization of the adherent or nonadherent lymphocytes after 5 days of culture was tested by measuring cytolysis of target fibroblasts or reticulum cells. The degree of binding or recognition of adsorbing fibroblast antigens can be expressed by the quotient of the cytotoxicity of the adhering and the nonadhering lymphocyte populations. This quotient is called the coefficient of adherence. The effect of serum factors on recognition was assayed by measuring the coefficient of adherence after the lymphocytes were preincubated for 30 min in EM + 50% of test serum.

RESULTS

Autosensitization against Autochthonous Thymus Reticulum Cells.—Our inbred rats appeared to be homozygous by in vivo skin grafting. Nevertheless, it was possible that undetectable minor antigenic differences between individual rats induced sensitization in vitro. To rule out this possibility, we investigated whether thymus lymphocytes could be autosensitized against reticulum cells derived from the same adult thymus gland. Whole thymus cultures were prepared from individual Lewis or BN rats. The thymus lymphocytes were separated from the reticulum cells after 5 days of culture and injected into footpads of syngeneic rats to test for autosensitization by the popliteal lymph node assay. We found (Table I) that the number of lymphocytes was

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TABLE I

Popliteal Lymph Node Reaction Produced by Thymus Lymphocytes Sensitized against Autochthonous Thymus Reticulum Cells

Rat thymus culture	Popliteal lymph node assay				
	Average cells ($\times 10^6$) per lymph node*		Ratio (right/left)	Relative lysis of target fibroblasts‡	
	Right	Left		Lewis	BN
Lewis	17	1	17	1.00	0.45
BN	24	3	8	0.43	1.00

* In syngeneic Lewis or BN rats.

‡ 5×10^6 lymphocytes from right lymph nodes were incubated with target fibroblasts for 65 h. To compute the relative lysis, the percent lysis of Lewis or BN fibroblasts was divided by the percent lysis produced by the specifically autosensitized syngeneic lymphocytes. The percent lysis of Lewis fibroblasts produced by Lewis lymphocytes was 73.5 ± 3.6, and that of BN fibroblasts by BN lymphocytes was 30.9 ± 3.6. These values were significantly greater ($P < 0.01$) than those produced by the allogeneic lymphocytes.

17 (Lewis) or 8 (BN) times greater in the popliteal lymph nodes draining the injection site of autosensitized compared with control thymus cells.

This increase in lymph node size appeared to indicate a GvH reaction similar to that obtained by the injection of parental lymphocytes into the footpads of F_1 hybrid rats (10). It has been shown that specifically cytotoxic lymphocytes develop in lymphoid organs involved in GvH reactions (13). Therefore, we assayed the ability of lymphocytes from these nodes to damage syngeneic or allogeneic target cells in vitro, to confirm that the increase in lymph node size represented a specific GvH reaction against self-antigens. We found (Table I) that the cytolysis of syngeneic fibroblasts was significantly greater than that of allogeneic fibroblasts. The immunospecificity of the lymph node assay indicates that immunocompetent thymus lymphocytes were induced to mediate an immune reaction against autochthonous reticulum cells originating from the same thymus gland. Hence, the thymus appears to contain T lymphocytes which can recognize strain-specific antigens that are truly syngeneic and probably accessible in vivo.

Kinetics of Autosensitization.—The absence of autosensitization in intact rats suggests that potentially self-reactive lymphocytes are inhibited by factors which are active in vivo, but which become inactive in cell culture. T cell autosensitization, like other cellular immune reactions, develops as a sequence of several stages, including recognition of antigen, differentiation and proliferation of lymphocytes, and mediation of immune effects. Therefore, autosensitization could be prevented in vivo by factors which might inhibit at least one of these stages. We used two approaches to identify the stages upon which natural inhibitors might act. We first studied the kinetics of autosensitization in vitro to discover the time when return of the lymphocytes to the rat could no longer

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suppress a GvH reaction. These studies are described here. The second approach, described below, was to study the kinetics of self-recognition. This enabled us to observe the inactivation of the inhibitory factors in vitro and provided a way to identify these factors.

To investigate the kinetics of autosensitization, we cultured lymphocytes on monolayers of syngeneic fibroblasts or thymus reticulum cells for various times in vitro, and injected them into the footpads of syngeneic rats. The popliteal lymph node assay was used to test the in vivo expression of autosensitization. Table II shows the results of one such experiment. Because of the relatively short incubation time, it was not necessary to include foreign serum in the culture medium. The lymphocytes together with the syngeneic fibroblasts were

TABLE II

Kinetics of Autosensitization of Lewis Thymus Lymphocyte Cells against Lewis Fibroblasts

Sensitization time in vitro	Popliteal lymph node assay		
	Average cells ($\times 10^6$) per right lymph node*	Lysis of target fibroblasts‡	
		Lewis	BALB/c
h			
0	3	11.9 ± 1.2	10.9 ± 0.4
2	3	9.1 ± 0.7	10.4 ± 0.6
6	8	17.0 ± 1.5	8.8 ± 1.7
24	12	21.6 ± 0.6	10.5 ± 2.0

* The control left lymph nodes contained an average of 2×10^6 cells per node.

‡ 5×10^6 lymphocytes from the right lymph nodes of each group were incubated with syngeneic Lewis or BALB/c mouse target cells for 65 h. The lysis of Lewis fibroblasts was significantly greater than that of the BALB/c controls at 6 and 24 h ($P < 0.01$).

collected at intervals by using a rubber policeman. The cell mixture was then injected into rat footpads. Lymphocytes and fibroblasts which were incubated together for 2 h or less in vitro did not produce a positive lymph node reaction. There was no significant increase in cell number, and lysis of syngeneic fibroblasts was not significantly different than the background lysis of BALB/c mouse fibroblasts. This indicates that syngeneic fibroblast antigens did not induce an immune response in vivo, even when close contact between lymphocytes and fibroblasts occurred in the footpad. However, an increase in lymph node size and immunospecific cytotoxicity was induced by the cells after they had been incubated for about 6 h in vitro. Thus, autosensitization appears to require a minimum period of induction that can take place in vitro within one cell generation. Moreover, once induction has occurred, an effect of autosensitization cannot be completely suppressed in vivo. Therefore, in vitro control of autosensitization does not appear to induce suppression of the differentiation, proliferation, or effector stages of the lymphocyte response.

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This suggests that effective inhibition of self-tolerant lymphocytes in vivo functions at the stage of recognition of self-antigens.

Receptors for Self-Antigens on Normal Lymphocytes.—The achievement of autosensitization in the absence of serum within 6 h suggests that recognition of self-antigens is an intrinsic property of some normal lymphocytes and does not require replication or mutation of the lymphocytes. To demonstrate the existence of receptors for self-antigens we used monolayers of syngeneic fibroblasts as immunoadsorbents for potentially self-reactive lymphocytes. Table III shows the results of an experiment in which BN lymph node cells were adsorbed on BN fibroblasts. The nonadherent lymphocytes were separated from those which adhered to the BN fibroblasts and were transferred to BN or Lewis fibroblast monolayers for sensitization for 5 days in vitro. Lysis of BN or Lewis target fibroblasts served as a measure of sensitization. We found that

TABLE III

Specific Recognition of Self-Antigens by Normal BN Lymph Node Cells

Adherence		Sensitization	
Adsorbing fibroblasts	Fraction of BN lymphocytes	Sensitizing fibroblasts	Percent lysis*
BN	Adherent	BN	22.3 ± 1.0
BN	Nonadherent	BN	8.8 ± 0.4
BN	Nonadherent	Lewis	26.0 ± 1.5
None	Unadsorbed	Lewis	23.3 ± 1.3

* 4×10^6 sensitized lymphocytes were incubated for 40 h with target fibroblasts of the same phenotype as the sensitizing fibroblasts.

the lymphocytes which adhered to syngeneic fibroblasts became autosensitized. The nonadherent BN lymphocytes were significantly less active ($P < 0.01$) in their ability to undergo autosensitization against BN fibroblasts. Nevertheless, such lymphocytes could be sensitized against Lewis fibroblasts to the same degree as unadsorbed BN lymphocytes sensitized directly against Lewis fibroblasts.

The immunospecificity of adsorption to syngeneic fibroblasts indicates that receptors for self-antigens exist on the surface of some normal lymph node cells. These receptors appear to characterize the fraction of lymphocytes in which autosensitization is induced in vitro.

Kinetics of Recognition of Self-Antigens.—The binding of lymphocytes to self-antigens appears to be the critical step in the induction of autosensitization (Table III). Hence, inhibition of autosensitization could be based upon regulation of this stage of primary recognition. The degree of specific binding to self-antigens can be expressed as the ratio of the lysis produced by the adherent lymphocytes to that produced by the nonadherent lymphocytes. This ratio, the adherence coefficient, serves as a measure of recognition. Factors which

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inhibit recognition of specific antigens can be detected by the degree to which they depress the coefficient of adherence.

The kinetics of lymphocyte adherence to self-antigens was compared with the kinetics of adherence to foreign antigens to learn whether potentially self-reactive lymphocytes are inhibited in their ability to recognize self-antigens at the time they are removed from the animals. It was found in previous studies that the coefficient of binding to foreign fibroblasts was maximal within 30–60 min (11). The kinetics of recognition of self-antigens, however, differed markedly. Recognition of self-antigens was minimal at 1 h and progressively increased at 3 and 5 h (Fig. 1). This indicates that potentially self-reactive lymphocytes are specifically inhibited in their ability to recognize self-antigens in vivo and that this inhibition is gradually lost in vitro.

Autologous Serum Inhibits Recognition of Self-Antigens.—The addition of the inhibitory factors to the adherence assay should suppress recognition of self-antigens and, therefore, decrease the coefficient of adherence. It was con-

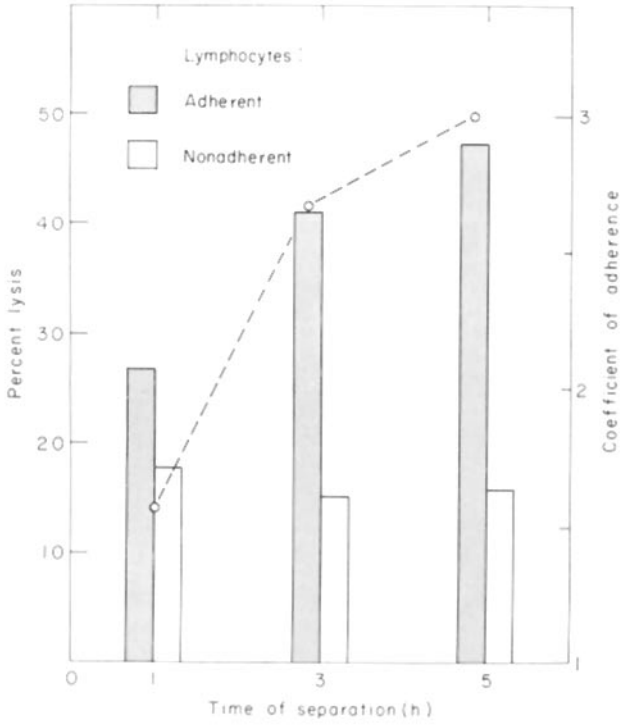

FIG. 1. Recognition of self-antigens on thymus reticulum cells. Normal Lewis lymph node cells were adsorbed on syngeneic reticulum cells for 1, 3, or 5 h. The adherent and nonadherent lymphocytes were separated and sensitized against the reticulum cells. Percent lysis of reticulum cells was measured after 44 h of incubation with 3×10^6 lymphocytes and the coefficient of adherence (○----○) was computed.

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ceivable that the inhibition of self-recognition was due to factors present in normal rat serum. To test this, we incubated lymphocytes with autologous rat or horse serum for 30 min and measured the coefficient of adherence at a time (3 h) when significant binding to self-antigens should take place. Table IV shows the results of two experiments in which the coefficient of adherence to syngeneic fibroblasts or thymus reticulum cells was measured after serum treatment. We found that specific binding to self-antigens was inhibited by 93 and 100% by pretreatment with autologous serum.

This result could be explained either by specific inhibition of self-recognition, by some general immunosuppressive action, or by a nonspecific toxic effect of rat serum on lymphocyte binding. To investigate these possible alternatives we studied the effects of autologous or allogeneic serum upon recognition of both foreign and self-antigens. Table V shows the results of these experiments.

TABLE IV

Autologous Serum Inhibits Recognition of Self-Antigens by Lewis Lymph Node Lymphocytes

Serum treatment	Adsorbing Lewis cells	Percent lysis by lymphocytes*		Coefficient of adherence	Percent inhibition‡
		Adherent	Nonadherent		
HS	Fibroblasts	17.0 ± 1.3	3.7 ± 0.8	4.50	
Lewis		12.8 ± 1.1	10.4 ± 0.1	1.23	93
HS	Reticulum	19.4 ± 1.3	9.3 ± 1.1	2.11	
Lewis		15.0 ± 0.1	15.5 ± 0.4	0.97	100

* Adherent or nonadherent Lewis lymphocytes were sensitized against Lewis fibroblasts or thymus reticulum cells. 3×10^6 sensitized lymphocytes were incubated with target cells for 40 h.

‡ Percent inhibition of adherence

$$= \frac{\text{Coefficient of adherence (HS)} - \text{Coefficient of adherence (Lewis serum)}}{\text{Coefficient of adherence (HS)} - 1} \times 100.$$

TABLE V

Effects of Autologous Serum on Recognition of Self- or Foreign Antigens

Serum treatment	Adsorbing fibroblasts	Percent lysis by lymphocytes*		Coefficient of adherence	Percent inhibition‡
		Adherent	Nonadherent		
HS	Lewis	11.7 ± 1.1	7.4 ± 0.4	1.60	
BN	Lewis	12.5 ± 0.8	8.2 ± 1.2	1.52	13
Lewis	Lewis	9.4 ± 0.5	8.6 ± 1.1	1.08	87
HS	C3H	38.4 ± 1.1	19.1 ± 0.6	2.01	
Lewis	C3H	46.4 ± 1.1	16.0 ± 1.3	2.91	<0

* 3×10^6 sensitized Lewis lymph node cells were incubated for 40 h with target cell of the same phenotype as the sensitizing fibroblasts.

‡ Compared with coefficient of adherence of the same fibroblasts produced by treatment with HS.

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Autologous serum decreased the coefficient of binding to Lewis fibroblasts by 87%. However, BN rat serum inhibited this binding by only 13%. The coefficient of adherence to C3H mouse fibroblasts was even greater after treatment with Lewis than with horse serum. Thus, inhibition of self-recognition by autologous serum in vitro appears to be immunologically specific. This suggests that autologous serum contains the factors which specifically regulate potentially self-reacting lymphocytes in vivo. These soluble serum factors appear to be lost or inactivated in vitro leading to recognition of self-antigens and induction of autosensitization.

Treatment of Adsorbing Fibroblasts Does Not Inhibit Their Recognition by Lymphocytes.—We pretreated Lewis fibroblasts with Lewis serum to confirm that autologous serum factors act on the lymphocytes and not on the adsorbing fibroblasts. Table VI demonstrates that recognition was not inhibited by this treatment.

TABLE VI

Effects of Treating the Adsorbing Fibroblasts with Autologous Serum

Serum treatment*	Adsorbing fibroblasts	Percent lysis by lymphocytes‡		Coefficient of adherence	Percent inhibition
		Adherent	Nonadherent		
HS	Lewis	31.2 ± 0.6	17.0 ± 1.5	1.85	
Lewis	Lewis	36.8 ± 1.1	20.0 ± 0.1	1.83	0

* The adsorbing Lewis fibroblasts were incubated with the serum for 30 min, and the serum was aspirated before the coefficient of adherence of Lewis lymph node cells was measured.

‡ 3×10^6 sensitized lymphocytes were incubated with Lewis target fibroblasts for 40 h.

DISCUSSION

The GvH and cytotoxic reactions described above are characteristic of cell-mediated immunity and are not likely to have been produced by antibodies. A T cell-specific marker is not yet available to identify the origin of rat lymphocytes. Nevertheless, the immunocompetent thymocytes which were sensitized against autochthonous thymus reticulum cells (Table I) or syngeneic fibroblasts (Table II) were most probably T lymphocytes. Immunospecific binding to syngeneic fibroblasts (Table III) indicated that a fraction of normal lymph node lymphocytes have specific receptors for self-antigens on their surface. Thus, normal rats appear to possess T lymphocytes which are potentially reactive with accessible self-antigens.

Recognition of self-antigens also has been detected in other systems. Boehmer and Byrd (14) found that mouse thymocytes can be stimulated by syngeneic spleen cells to synthesize DNA in a mixed lymphocyte reaction. Micklem and his co-workers (15) found that mouse lymphocytes form clusters (rosettes) with syngeneic or autochthonous erythrocytes. Although the potential immune

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effects of such lymphocytes were not studied, these observations are compatible with our findings and support the concept that self-tolerant lymphocytes exist in normal animals.

The question of the elimination or persistence of lymphocytes in artificially induced tolerance has been studied by a number of investigators. It appears that tolerant lymphocytes do persist after the induction of tolerance to foreign antigens. Antigen-binding lymphocytes have been found in animals made tolerant to thymus-dependent as well as thymus-independent antigens (16–19). Both natural and artificial tolerance, therefore, may be characterized by the persistence of lymphocytes with receptors which can bind the specific tolerogenic antigens.

The functional reactivation of tolerant lymphocytes provides additional evidence for this concept. Chiller and his colleagues found kinetic differences in artificial tolerance between thymus and bone marrow (B) cells (20). Nevertheless, both T and B cell tolerance appears to be reversible. McGregor, McCullagh, and Gowans were able to reactivate tolerant thoracic duct lymphocytes against sheep erythrocytes by incubating the lymphocytes, including T cells, in vitro (21). Sjöberg found that B cell tolerance against a thymus-independent antigen could be reversed by incubating the lymphocytes in vitro (22). Reversal of tolerance in vitro by removal of a tolerizing protein antigen was also demonstrated by Byers and Sercarz (23). These examples of the breakdown of artificial tolerance cannot be explained by immunization against cross-reacting antigens or new carrier determinants (24). Hence, they are consistent with our demonstration of the activation of self-tolerant T lymphocytes in vitro.

We used two approaches to identify the factors that probably function to inhibit autosensitization in vivo. The kinetics of autosensitization using the popliteal lymph node reaction indicated that suppression of self-reactive lymphocytes in vivo was lost after the recognition of self-antigens in vitro (Table II). Study of the kinetics of recognition of self-antigens (Fig. 1) suggested that reversal of self-tolerance in vitro was due to the progressive loss of factors which inhibit recognition of self-antigens by potentially self-reactive T lymphocytes. Thus, the results of both types of experiments suggest that critical control of autosensitization in vivo involves suppression of recognition.

We were able to restore the inhibition of self-recognition in cell culture by pretreating the lymphocytes with autologous serum (Table IV). Recognition of foreign antigens was not blocked by this treatment (Table V). Therefore, the factors which specifically prevent self-recognition and autosensitization in vivo appear to be present in normal serum.

Serum factors also have been described which suppress cell-mediated reactions in tolerance induced to allogeneic antigens. Wegmann and his co-workers found that serum from tetraparental mice inhibits the in vitro cytotoxicity produced by mutually incompatible lymphocytes in chimeric tetraparental

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mice (25). Tolerogenic antigen alone could not account for this blocking effect because serum from the parental strains or from their F_1 hybrids did not block cytotoxicity. Wegmann et al. speculated that antibody or antigen-antibody complexes were the blocking agents. Similar antibody-like blocking factors have been detected in the sera of mice in which neonatal tolerance was induced to allogeneic transplantation antigens (26). Voisin and his colleagues have partially characterized certain immunoglobulin fractions associated with a similar form of tolerance (27).

The relationship of these antibody-like factors to the normal serum factors which inhibit self-tolerant T cells is not clear. Our factors may be soluble nonimmunogenic self-antigens since the lymphocytes, but not the adsorbing fibroblasts, respond to treatment (Table VI). These tolerogenic self-antigens may be shed from the lymphocytes in vitro because of their low affinity for surface receptors, or they might be lost during the rapid turnover of membrane proteins (28). Soluble antigens alone can inhibit the recognition of xenogeneic antigens in our system (H. Wekerle, manuscript in preparation). However, antigen-antibody complexes (29) as well as antibody to self-receptors (30) are among the possibilities that cannot be ruled out at this time.

The factors which inhibit self-recognition appear to function as natural T cell tolerogens. Hence, their characterization could possibly elucidate the molecular basis of T cell tolerance. Preliminary studies indicate that the factors are labile. Inhibitory activity is lost after the serum is frozen ($-20°C$) or incubated (37°C) for several hours.

It is possible that additional mechanisms operate to ensure self-tolerance to certain antigens. We have been unable to induce autosensitization in vitro using lymphocytes themselves as stimulator cells (I. R. Cohen, manuscript in preparation). In addition, lymphocytes from animals with specifically induced tolerance have failed to demonstrate mixed lymphocyte reactions in vitro (31). It has been shown that adherent cells or their products are required for mixed lymphocyte reactions between purified populations of allogeneic lymphocytes (32). Hence, recognition of antigens on lymphocytes themselves may be regulated by mechanisms such as special immune response genes (33) or perhaps by controlling cells (34–36).

It has been reported that mixed lymphocyte reactions may be stimulated by autologous lymphocytes that have been transformed into cell lines (37). However, it is difficult to relate these findings to true autosensitization since transformation into cell lines may involve the expression of viral or other abnormal antigens.

The results of our earlier studies of autosensitization raised doubts regarding the general applicability of the hypothesis that natural self-tolerance is based upon the elimination of potentially self-reactive lymphocytes (3–5). The experiments presented in this paper confirm the existence of self-tolerant lymphocytes in the thymus and lymph nodes of normal adult rats. Nevertheless, it is

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conceivable that in vitro culture of syngeneic fibroblasts or thymus reticulum cells alter self-antigens so that they appear "foreign" to autochthonous lymphocytes. The development of GvH reactions in vivo suggests that the self-antigens which induce sensitization are also present in vivo. However, the most convincing evidence against the elimination hypothesis is the demonstration of a mechanism that specifically inhibits self-recognition. The existence of such an inhibitory mechanism in fresh normal serum indicates that the specific self-antigens really exist in vivo. Had these antigens been an artifact of cell culture, there would have been no need for a natural mechanism to suppress their recognition. Hence, the elimination theory of natural tolerance, despite its appealing logic, cannot account for the experimental evidence. Our results indicate that tolerance to certain self-antigens is probably based upon regulation of self-recognition. Further studies are needed to investigate the implications of our findings to three general areas: the regulation of the immune reactivity of T cells in transplantation, the importance of regulation in autoimmune diseases, and the possible physiologic function of lymphocytes that have receptors for self-antigens.

SUMMARY

We studied the mechanisms underlying the natural tolerance of thymus-derived (T) lymphocytes for self-antigens. Lymphocytes from the thymus or lymph nodes of inbred rats were autosensitized in vitro against monolayers of autochthonous thymus reticulum cells or syngeneic fibroblasts. Receptors for self-antigens were detected by the specific adherence of normal lymphocytes to syngeneic cells. The achievement of active cell-mediated autosensitization was assayed by measuring the immunospecific lysis of syngeneic target cells in vitro, or graft-*versus*-host (GvH) reactions in vivo. The following observations were made using these systems.

(*a*) A fraction of normal lymphocytes was found to have specific surface receptors that are able to recognize self-antigens which seem to be accessible in vivo. These potentially self-reactive lymphocytes were activated by incubation with syngeneic or autochthonous cells in vitro. Hence, the elimination of potentially self-reactive lymphocytes cannot be the only basis for natural self-tolerance. Therefore, the maintenance of self-tolerance in vivo appears to involve suppression of the immune reactivity of such self-tolerant lymphocytes.

(*b*) We found that control of autosensitization depends upon the inhibition of the recognition of self-antigens. A GvH reaction in vivo could not be suppressed once recognition of self-antigens had occurred in vitro. Moreover, studies of the kinetics of antigen recognition indicated that several hours of incubation in vitro were needed for the inactivation of factors specifically inhibiting self-recognition.

(*c*) We found that factors which inhibit self-recognition are present in fresh

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autologous serum. Treatment of the lymphocytes, but not syngeneic adsorbing cells, with autologous serum prevented recognition of syngeneic antigens. Allogeneic serum did not prevent self-recognition, and autologous serum did not inhibit the recognition of foreign antigens.

These findings indicate that natural tolerance of T lymphocytes to self-antigens can be regulated by serum factors which act on the lymphocytes. The immunospecificity of the inhibitory effect suggests that these factors may be soluble self-antigens in a tolerogenic form.

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