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Abstract. *Thymus lymphocytes of normal adult rats were autosensitized in vitro against soluble antigens extracted from the brains of syngeneic rats. Injection of the autosensitized lymphocytes into syngeneic rats led to the development of brain lesions suggestive of autoimmune encephalomyelitis. Injection of control lymphocytes or the antigen extract alone did not cause lesions. Since sensitization in vitro requires the presence of lymphocytes programmed with specific receptors, the results indicate that normal rats have lymphocytes capable of recognizing central nervous system self-antigens. Hence, regulatory mechanisms, inoperative in vitro, probably function in vivo to prevent immune activation of self-recognizing lymphocytes and autoimmunity. This concept suggests a new approach to exploring the pathogenesis of autoimmune diseases.*

Autoimmune diseases are caused by an attack of the immune system against the body's own constituents (self-antigens). Clinically, many autoimmune diseases appear to involve damage to specific organs or tissues such as the thyroid gland in Hashimoto's thyroiditis (*1*), the skeletal muscles in polymyositis (*2*), or the mucosa of the large bowel in ulcerative colitis (*3*). Although autoantibodies reactive with specific self-antigens are often demonstrable in these diseases, clinical and experimental evidence indicates the immune damage may be mediated by autoreactive thymus-derived (T) lymphocytes rather than by autoantibodies (*1–3*).

Allergic encephalomyelitis is an experimental model of an autoimmune disease in which the target is the central nervous system (*4, 5*). This disease can be produced in rats, guinea pigs, or monkeys by injecting animals with an antigen extracted from the central nervous system together with adjuvant materials such as complete Freund's adjuvant. The active encephalitogenic antigen has been identified as basic protein, a positively charged protein normally found in the myelin sheath that insulates the axons of the central nervous system (*6, 7*). The autoimmune reaction to basic protein results in a demyelinating encephalitis characterized by lymphocytic infiltration and inflammation of the central nervous system, which may progress to clinical paralysis and death. The disease appears to be mediated by the T lymphocyte system, since it can be passively transferred to normal animals by immune lymphocytes but not by serum antibodies (*4, 5, 8*). In fact, specific antibodies may actually protect against the disease, presumably by binding to basic protein and inhibiting the access of autoreactive T lymphocytes (*4, 5, 9*).

The human counterpart of experimental allergic encephalomyelitis appears to be postvaccinal encephalomyelitis, a rare complication of vaccination with rabies virus grown on central nervous system tissues (*10*). Potentially more important, although more tenuous, is the suggestion that a similar autoimmune process may underlie some cases of the demyelinating disease multiple sclerosis (*10*).

The immune system is normally tolerant of self-antigens. Therefore, autoreactive lymphocytes must develop because of some defect in the physiologic mechanisms which function to ensure self-tolerance. Hence, the nature of self-tolerance is an important consideration in understanding autoimmunity.

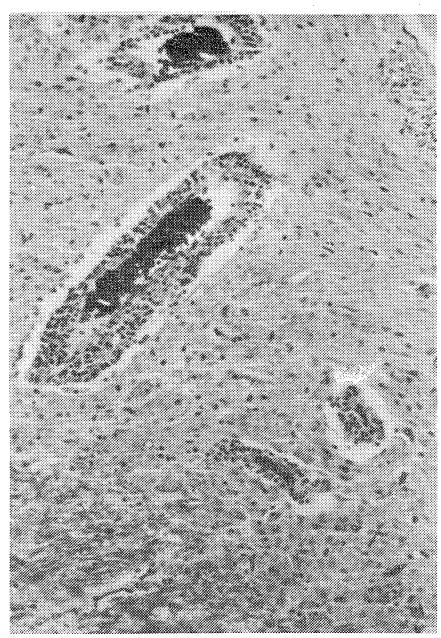

Fig. 1. Lesions of encephalomyelitis in the medulla of a Lewis rat. The blood vessels, in cross section, are surrounded by a heavy infiltrate of lymphocytes. These lesions were produced by lymphocytes sensitized against syngeneic central nervous system antigen in vitro (Table 1, group 1). Similar lesions were produced by active immunization in vivo with antigen and adjuvant (*19*).

Burnet proposed that lymphocytes potentially reactive with self-antigens are eliminated during a critical period in the ontogeny of the immune system (*11*). The process of elimination or irreversible inactivation was thought to be triggered by contact between self-antigens and immature lymphocytes that carry complementary membrane-bound receptors. Hence, the self-antigens themselves select against lymphocytes capable of self-recognition. Burnet postulated that only after maturation are lymphocytes activated rather than killed by contact with antigen. However, no "forbidden clones" of potentially self-reactive lymphocytes normally survive the period of elimination, and the animal is immunologically tolerant of its self-antigens.

According to this elimination theory, autoimmune diseases are caused either by somatic mutation of lymphocytes into self-reactive cells or by changes in self-antigens so that they are recognized by receptors of mature lymphocytes (*11*). Experimental allergic encephalomyelitis, postvaccinal encephalomyelitis, and possible other demyelinating conditions were thought to result from exposure of mature lymphocytes to antigens that are normally sequestered within the central nervous system (*12*). It was supposed that basic protein is not accessible to lymphocytes during their critical period of development and, therefore, potentially reactive lymphocytes escape elimination. However, it is difficult to imagine that basic protein or other actively turned-over substances are completely sequestered for the lifetime of a normal individual (*4*). Other theories suggest that the appearance of organ-specific autoreactive lymphocytes is related to immunization against antigens that cross-react with self-antigens (*13*) or to intense proliferation of lymphocytes stimulated by adjuvants (*4*).

The notion of elimination of forbidden clones of lymphocytes, however, was recently challenged by the results of studies in this laboratory. We found that the thymus, spleen, and lymph nodes of normal adult rodents are populated with lymphocytes that can recognize syngeneic fibroblasts or syngeneic or autochthonous thymus reticulum cells in vitro (*14–16*). Recognition of these self-antigens led to immune activation of the lymphocytes and to the development of specific autoreactive effector lymphocytes either in vitro or

in vivo. Activation of potentially self-recognizing lymphocytes appeared to be prevented in the intact animal by the presence of factors in the serum (*15, 16*). These factors behaved as self-antigens in a soluble, nonimmunogenic form. They appeared to combine with specific lymphocyte receptors and to block them from being activated by immunogenic forms of the same self-antigens. These findings suggest that tolerance to some self-antigens on fibroblasts or reticulum cells is based on the regulation of recognition rather than on the elimination of lymphocytes.

These observations suggest that true tissue-specific autoimmunity might also be based on the immune activation of existent potentially self-recognizing lymphocytes, rather than on the appearance of new forbidden clones. We now report the results of experiments designed to ascertain whether lymphocytes that recognize central nervous system antigens exist and can be activated. Our general approach was to culture rat lymphocytes with central nervous system antigens in vitro, inject the lymphocytes intravenously into syngeneic rats, and observe the rats for evidence of encephalomyelitis. The appearance of tissue-specific autoimmunity would indicate the preexistence of lymphocytes capable of recognizing and reacting against self-antigens.

We used lymphocytes obtained from the thymus glands of normal adult male Lewis rats (*17*) to exclude as much as possible contamination with thymus-independent B lymphocytes. The central nervous system antigen was extracted in phosphate-buffered saline, *p*H 7.2, from the cerebellums and spinal cords of syngeneic Lewis rats (*18*). The encephalitogenicity of the antigen was proved by its ability to cause paralysis and histologic lesions diagnostic of experimental encephalomyelitis 11 to 21 days after injection with adjuvant into Lewis rats (*19*). Autosensitization against the central nervous system antigen was induced by incubating thymus lymphocytes with the test antigen in Dulbecco's modification of Eagle's medium without serum or adjuvants added, as described (*20*). The cultures contained a feeder monolayer of syngeneic fibroblasts (*16*) to facilitate autosensitization (Table 1). Lesions consisting of perivascular infiltration of lymphocytes and breakdown of myelin (Fig. 1) were found only in the groups of rats that received thymus lymphocytes cultured with central nervous system antigen for 18 hours at 37°C (Table 1, group 1). These lesions were prominent in the cerebellum and medulla, but were also found in the spinal cord. Control groups consisted of rats injected with lymphocytes that were cultured with antigen under conditions of time and temperature not sufficient for active sensitization. We found in earlier studies that about 6 hours of culture at 37°C is necessary for the induction of sensitization in vitro of thymus lymphocytes (*16*). Thus, groups 2 and 3 (Table 1) which were injected with lymphocytes exposed to antigen at 4°C, or 37°C for only 1 hour, failed to show central nervous system lesions. Indeed, injection of medium containing free antigen that had been incubated with lymphocytes also failed to sensitize the recipient rats (group 4). In addition, thymus lymphocytes cultured with syngeneic fibroblasts without added antigen did not produce encephalomyelitis (group 5). All rats were injected intravenously with pertussis vaccine. The negative results of the control groups argue against the possibilities that the central nervous system extract was modified by lymphocyte enzymes or other means to become immunogenic in vivo for Lewis rats, either by itself or passively adsorbed onto the injected lymphocytes, or that culture of lymphocytes in vitro led to a nonspecific artifact. Therefore, we may conclude that the lesions were mediated by thymus lymphocytes sensitized in vitro specifically against syngeneic central nervous system antigen.

Table 1. Encephalomyelitis produced by Lewis thymus lymphocytes sensitized against syngeneic central nervous system antigen in vitro. Suspensions of thymus lymphocytes were obtained by pressing thymus glands of Lewis rats (6 to 8 weeks old) through a fine wire mesh. The lymphocytes (10^8 in 10 ml) were cultured in Eagle's medium without serum for 1 or 18 hours at 37° or 4°C on monolayers of Lewis fibroblasts (*16*). Central nervous system antigen (3 mg of antigen protein per 10^8 lymphocytes) was added to groups 1 to 3. The lymphocytes were collected, washed (three times) in phosphate-buffered saline (PBS) by centrifugation, and injected intravenously into male Lewis rats. Each rat received 550×10^6 lymphocytes in 3 ml of PBS. The rats in group 4 were injected with 3 ml of the cell-free medium removed from group 1. All the recipient rats were injected intravenously with 0.1 ml of the pertussis vaccine (*19*) at the same time that they received test lymphocytes or culture medium. After 10 days, the brains of the recipient rats were fixed in formalin, and sections were stained with luxol fast green. The slides were coded and examined for evidence of perivascular cuffing of lymphocytes (Fig. 1) or degeneration of myelin (*24*).

Group	Sensitization culture	Diseased/ normal rats
1	37°C, 18 hours	5/6
2	4°C, 18 hours	0/17
3	37°C, 1 hour	0/11
4	Cell-free medium of group 1	0/19
5	37°C, 18 hours, no antigen	0/3

Sensitization in vitro was done in the absence of adjuvants, in a closed system in which there was no entry of stem cells from without, and in which spontaneous cell replication was unlikely during an 18-hour period. We have found that primary sensitization in such in vitro systems depends upon the preexistence of lymphocytes with specific receptors for the sensitizing antigens (*15, 16*). Recognition of specific antigen induces sensitization of these lymphocytes, which begin to proliferate after about 72 hours (*21*). Therefore, successful sensitization against syngeneic central nervous system antigen in vitro indicates that competent lymphocytes exist which bear receptors for this antigen in mature rats. Furthermore, these lymphocytes, once they are triggered in vitro, produce inflammatory lesions in the central nervous system, either by themselves or by recruiting specific effector lymphocytes (*20*).

The central nervous system antigen used in these studies was not purified. Hence we cannot be certain that the active immunogen was basic protein until further investigation of this immunogen is completed. Nevertheless, our evidence supports the conclusion that lymphocytes recognize and are activated against central nervous system antigen.

These results are in conflict with the notion that tolerance to self-antigen must be based on elimination of self-reactive lymphocytes (*11, 12*). However, they are compatible with our earlier observation that lymphocytes exist with receptors for self-antigens on fibroblasts or thymus reticulum cells (*14–16, 20*). Indeed, normal as well as immune lymphocytes were observed to bind basic protein antigen, presumably by way of antibody-like receptors (*22*). Hence, tolerance to central nervous system antigen as well as to syngeneic fibroblasts or reticulum cells must depend on mechanisms which regulate the immune activation of potentially self-recognizing lymphocytes in vivo (*15, 16*).

The nature of these mechanisms are unknown. However, the results of studies of the regulation of autosensitization against syngeneic fibroblasts sug-

gest that a soluble, nonimmunogenic form of self-antigen might have a role. Such a nonimmunogenic self-antigen could produce tolerance by occupying specific lymphocyte receptors and preventing their activation by immunogenic self-antigen. Tolerance to basic protein would, therefore, depend upon a balance between the concentration of the immunogenic and nonimmunogenic forms of the antigen. This concept is supported by the finding of Teitelbaum and co-workers that allergic encephalomyelitis can be suppressed, or even cured, by injecting animals with a low-molecular-weight basic copolymer of amino acids (*7*). The copolymer was observed to cross-react with lymphocytes previously sensitized to encephalitogenic basic protein (*23*). Hence it is conceivable that the copolymer suppresses encephalomyelitis by binding to specific lymphocyte receptors and preventing recognition of immunogenic basic protein. Our findings implicating nonimmunogenic self-antigen in the regulation of autosensitization against fibroblast antigens (*15, 16*) would suggest that a nonimmunogenic fragment of basic protein may exist in vivo and prevent activation of lymphocytes capable of recognizing immunogenic basic protein.

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17. The Lewis rats, originally obtained from Microbiological Associates (Baltimore), were continually inbred and were tested for homozygosity by Dr. A. Meshorer of the Animal Breeding Center of this institute. Rats aged 4 to 6 weeks were used for the experiments.
18. The central nervous system antigen was prepared by homogenizing with a glass piston the cerebellums and spinal cords of 20 rats in about 20 ml of phosphate-buffered saline at room temperature. The particulate matter was removed by centrifuging the homogenate for 20 minutes at 3000 rev/min. The supernatant fluid was collected and lyophilized. The protein concentration was determined by the biuret reaction. The extract was dissolved in water for use in experiments.
19. Central nervous system antigen (1.2 mg of protein in 0.2 ml of H_2O) was emulsified with 0.2 ml of complete Freund's adjuvant (Difco, Detroit) and injected into the hind foot pads of six Lewis rats. The animals received in the dorsum of the hind feet 0.1 ml of pertussis vaccine (Rafa, Jerusalem) containing 24×10^6 microorganisms (H. C. Rauch, personal communication). Within 11 to 21 days all of the six Lewis rats developed paralysis. Histological lesions diagnostic of encephalomyelitis were observed in all the animals.
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24. Dr. U. Klopfer of the Kimron Veterinary Institute (Israel) examined the slides.
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