Article abstract—In adult strain 13 guinea pigs, two lines of evidence show that there are natural autoreactive thymocytes that can react with myelin basic protein (BP) or the encephalitogenic nonapeptide (EP). An autoradiographic binding assay revealed antigen-specific receptors for ^{125}I-BP on thymocytes. A ^{3}H-thymidine antigen-specific proliferation assay demonstrated that normal thymocytes were activated by EP- or BP-pulsed macrophages. Soluble BP suppressed the activation of thymocytes by macrophage-associated BP. The mode of presentation of BP or EP, whether macrophage-associated or soluble, may be critical in maintaining self-tolerance and preventing an autoimmune attack on the central nervous system.

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Natural occurrence of thymocytes that react with myelin basic protein

Lawrence Steinman, M.D., Irun Cohen, M.D., and Dvora Teitelbaum, Ph.D.

Autoimmune diseases are caused by an attack of the immune system against the body's own constituents—self-antigens. Burnet[1] attributed self-tolerance to the elimination of forbidden clones of autoreactive lymphocytes. Autoimmune disease was viewed as a consequence of failure to remove these forbidden clones, somatic mutation of lymphocytes into self-reactive cells, or chemical change in a self-antigen, which would allow its recognition by receptors of mature lymphocytes.[2] Cohen and Wekerle[3] provided early evidence that T cells with receptors for self-antigen were present in normal animals. Others[4,5] demonstrated that normal T lymphocytes could react to self-antigens of the major histocompatibility complex when these antigens were modified by viruses or synthetic chemical moieties.

If self-tolerance is not due to absence of autoreactive clones, dynamic mechanisms must regulate the expression of lymphocytes directed at self-antigens. These concepts may be related to the understanding of demyelinating diseases. Injection of guinea pigs with myelin basic protein (BP) or the encephalitogenic nonapeptide (EP), comprising residues 114 to 122 of BP,[6,7] in complete Freund's adjuvant, induces experimental allergic encephalomyelitis (EAE).[8] We studied the specificity of thymocyte binding of BP and EP. We then investigated how T-cell responses to BP may be regulated, utilizing an earlier observation that the mode of antigen presentation may be a critical factor in maintaining self-tolerance.[9-11]

Materials and methods. *Animals.* Strain 13 guinea pigs weighing 300 to 500 gm were obtained from the Experimental Animal Center of the Weizmann Institute.

Antigens. Guinea pig BP was purified from guinea pig spinal cord by delipidation, acid extraction, and column chromatography of sulfoethyl-Sephadex.[12] The encephalitogenic nonapeptide (EP) comprising residues 114 to 122 of bovine BP, Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys, was synthesized by the Merrifield solid-phase technique,[13] purified as described by Eylar and associates,[14] and checked by amino acid analysis. Poly (Glu^{50}, Tyr^{50}), abbreviated poly (G, T), was synthesized according to the method of Katchalski and Sela.[15] Lysozyme was purchased from Worthington Biochemical Co., Freehold, NJ.

Cell culture medium. Minimal essential medium for suspension (MEM-S) with added nonessential

From the Department of Neurology (Dr. Steinman), Stanford University, Stanford, CA, the Department of Cell Biology (Dr. Cohen), and the Department of Chemical Immunology (Dr. Teitelbaum), Weizmann Institute of Science, Rehovot, Israel.

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Address correspondence and reprint requests to Dr. Steinman, Department of Neurology, Stanford University, Stanford, CA 94305.

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amino acids; glutamine, penicillin, and streptomycin solutions; and RPMI-1640 medium were obtained from Microbiological Associates, Jerusalem, Israel. Normal guinea pig serum was obtained from Grand Island Biological Co., Grand Island, NY.

Thymocyte suspension. Thymuses were excised with special care to dissect mediastinal lymph nodes. The thymus was teased in MEM-S. Cells were washed in the same medium, and dead cells were removed by the Ficoll-Hypaque method. Thymocytes, greater than 99% viable, were resuspended at desired concentrations.

Autoradiography. Previously described techniques were used.[17,24] BP was labeled with ^{125}I by the chloramine-T method. The specific activity ranged from 4 to 8 μCi per microgram. Unlabeled antigen in 500 molar excess in 0.1-ml MEM-S was added to the Ficoll-Hypaque purified thymocytes for 30 minutes at 37°C. Labeled antigen, 0.1 ml (5 μg per milliliter), was then added to the thymocytes for another 30 minutes at 37° C. The cells were washed three times by centrifugation, and smears were prepared for autoradiography. The emulsion (Ilford.k5, Essex, England) was exposed for 10 days. The cells were counted under ×1000 magnification; 10 grains per cell was regarded as positive. At least 4000 cells were counted on each slide.

Preparation of adherent monolayers. Peritoneal exudate macrophages were prepared from unprimed strain 13 guinea pigs 4 days after intraperitoneal injection of 10 ml of sterile mineral oil (Drakeol, Butler, PA) by lavaging the peritoneum with sterile phosphate-buffered saline plus heparin, 5 units per milliliter. All procedures were then performed in RPMI plus 25 mM N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid (Hepes) buffer without serum. Peritoneal exudate cells (20×10^6) were allowed to adhere to 100-mm Petri dishes for 2 hours at 37°C and then washed to remove nonadherent cells.

Sensitization to antigen in vitro. Macrophages were pulsed for 1 hour with 100 μg of either BP, EP, or (G,T) per ml at 4°C. Antigen not associated with macrophages was removed by changing the medium three times. Unprimed thymocytes (100×10^6) were then added to the macrophage monolayers. After 16 hours of incubation of the thymocytes on the macrophage monolayers at 37° C, nonadherent cells were removed, then replated for 1 hour to readhere detached macrophages. Cells were more than 98% viable by trypan blue exclusion.

Recruitment of lymphocytes in vivo. Thymocytes were then injected into the footpads of syngeneic animals at a concentration of 20×10^6 cells in 0.1 ml of phosphate-buffered saline.

Lymphocyte transformation. After 11 days, the draining popliteal nodes were excised and lymph node cell suspensions were prepared. After 1 hour of adherence on plastic Petri dishes to remove debris and dead cells, 5×10^5 cells were placed in culture on Greiner microtiter plates. Medium for the lymphocyte transformation phase consisted of RPMI plus 25 mm Hepes plus 1.25% fetal calf serum (Rehatuin, Phoenix, AZ). Unprimed, unstimulated syngeneic peritoneal exudate macrophages (5×10^4) were irradiated with 2000 rad and added to each culture. Cultures were then incubated with 50 μg per milliliter of various test antigens for 72 hours. Then 1 μCi of (^3H-) thymidine (Israel Atomic Energy Commission, Negev, Israel) was added for 8 hours. Cultures were then harvested on a MASH-2 cell harvester, and radioactivity was determined in scintillation fluid in a Packard B counter. Results are expressed as cpm ± SEM for triplicate cultures, or stimulation index (SI) = (cpm incubation with test antigen)/(cpm incubation with no antigen).

Results. *Autoradiographic demonstration of antigen-specific thymocyte binding to ^{125}I-BP.* The number of antigen-binding thymocytes from unprimed strain 13 guinea pigs is shown in table 1. Inhibition of binding of ^{125}I-BP was carried out at

Table 1. Specificity of binding of ^{125}I-BP to strain 13 guinea pig thymocytes: Competition with 500-fold molar excess of unlabeled antigens*

Unlabeled antigen	Binding cells/10^4	% inhibition	Cross-reactivity
None	33		
BP	5	85	+
EP	15	55	+
Lysozyme	36	0	−

* Cells were counted under × 1000 magnification; 10 or more silver grains per cell was regarded as positive.

37° C in the presence of a 500-fold molar excess of competing antigen. Unlabeled BP, and to a lesser extent EP, effectively competed with ^{125}I-BP in antigen binding to thymocytes. Lysozyme, a basic protein of similar molecular weight to BP, did not inhibit ^{125}I-BP binding at all. Most binding cells showed a high density of grains over one pole of the cell in a "capped" distribution[17] (figure).

Sensitization of normal thymocytes to myelin antigens. In order to determine whether normal thymocytes could be sensitized to BP, we utilized an in vitro sensitization system. In this scheme, we could focus on the initial phase of thymocyte recognition and separate this process from the subsequent differentiation of effector lymphocytes. A three-phase experimental format was used, as described.[9-11,16] First, thymocytes were sensitized in vitro on BP-, EP-, or (G,T)-pulsed macrophages. Second, the sensitized T-cells were injected into the connective tissue spaces of the hind footpads of syngeneic strain 13 guinea pigs. The sensitized lymphocytes migrate to the regional popliteal node, where they recruit effector T lymphocytes. These two cell types—one involved in the afferent or sensitization phase of the immune response and the other in the efferent or effector phase—have been designated the initiator T lymphocyte (ITL) and the recruited T lymphocyte (RTL).[9,16] Third, the immunospecific ^{3}H-thymidine proliferative response of the RTL serves to indicate whether ITLs were triggered in vitro.

Both BP and EP associated with macrophages could be recognized by normal thymocytes (table 2). The responses were specific in that there was cross-reactivity between EP and BP, whereas there was no cross-reactivity with either of these antigens and the unrelated synthetic peptide (G,T). Thus, ITL sensitized to EP macrophages recruited comparable responses to EP and BP but induced no response to (G,T). ITL sensitized on BP macrophages recruited a 4.1-fold response to BP and a 2.4-fold response to EP but no response to (G,T). (G,T)-macrophage-sensitized ITL recruited RTL capable of responding to (G,T) (SI = 5.1) but not to BP. The possibility that detached macrophage-antigen complexes alone could recruit significant RTL responses seems unlikely from previous studies.[9,16]

Soluble BP inhibits activation of ITL on BP macrophages. We next evaluated the possibility that soluble BP might inhibit the activation of thymic ITL by macrophage-bound BP, as shown previously with splenic ITL.[9] Indeed, soluble BP, present during ITL sensitization, reduced the subsequent RTL response to BP from an SI of 3.4 to 2.3 ($p < 0.01$) (table 3). When soluble (G,T) was present during sensitization of ITL to BP macrophages (group 3), there was no inhibition of sensitization to BP and only a low-level recruitment of RTL to (G,T), SI = 2.1. Inhibition by BP cannot be due to a direct cytotoxic effect of soluble BP at 100 μg per milliliter, and soluble BP did not block sensitization of ITL to (G,T)-pulsed macrophages.[9]

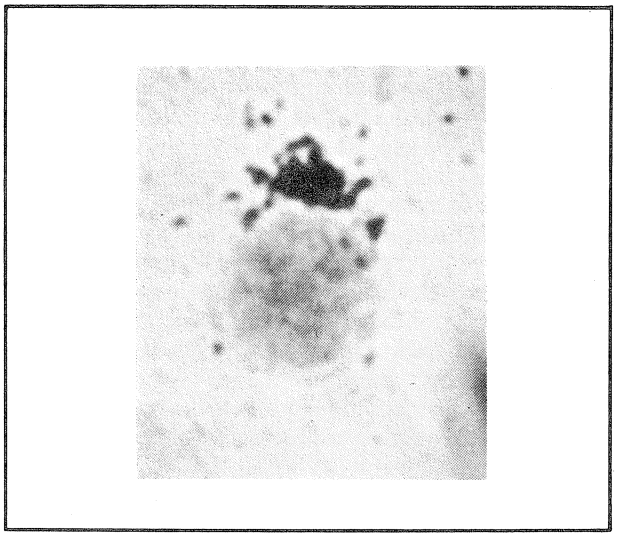

Figure. Binding of ^{125}I-BP to thymocyte. Radioautograph reveals typical distribution of grains over one pole of cell. × 9500.

Table 2. Specificity of RTL response to myelin antigens*

Injected ITL sensitized in vitro on	Response of RTL to various antigens (cpm) [S.I.]†			
	None	EP	BP	(G,T)
EP macrophage	522 ± 59	2089 ± 171 [4.0]	1644 ± 88 [3.1]	550 ± 147 [1.0]
BP macrophage	292 ± 49	718 ± 61 [2.4]	1185 ± 148 [4.1]	228 ± 37 [0.8]
(G,T) macrophage	198 ± 12	N.T.	328 ± 47 [1.7]	1013 ± 101 [5.1]

* Strain 13 guinea pigs were injected in both hind footpads with sensitized thymic ITL (20×10^6). Eleven days later, RTL from the draining lymph nodes were cultured with soluble EP, BP, or (G,T), all at 50 μg per milliliter, and their thymidine incorporation was measured.
N.T. = not tested.
† S.I. = stimulation index.

Table 3. Effect of soluble BP on activation of ITL by BP-pulsed macrophages*

Group	Injected ITL sensitized in vitro on	Response of RTL, cpm ± SEM (S.I.) None	BP	(G,T)
1	BP macrophages	463 ± 36	1551 ± 104 (3.4)	414 ± 16 (0.9)
2	BP macrophages plus soluble BP	272 ± 85	694 ± 194 (2.3)	319 ± 91 (1.2)
3	BP macrophages plus soluble (G,T)	435 ± 38	2438 ± 764 (5.6)	901 ± 76 (2.1)

* Strain 13 guinea pigs were injected in both hind footpads with 20 × 10^6 thymic ITL that had been incubated with BP macrophages (group 1) or BP macrophages in the presence of either 100 μg per milliliter soluble BP (group 2) or 100 μg per milliliter soluble (G,T) (group 3).

Discussion. EAE has been studied as a model for demyelinating disease. EAE results from the activation of T lymphocytes sensitized to BP or the encephalitogenic fragment,[6-8,18,19] but a critical factor is the mode of presentation of BP. BP can either induce EAE when associated with complete Freund's adjuvant or suppress EAE when in soluble form or associated with incomplete adjuvant.[20,21] These studies demonstrated that thymus-derived lymphocytes could be triggered by BP bound to syngeneic macrophages, whereas soluble BP inhibited this activation.

The present radioautographic studies demonstrated that thymic lymphocytes can specifically bind BP. Earlier studies showed that BP was bound by T cells from rat strains susceptible to EAE[18] and by unfractionated peripheral lymphocytes from guinea pigs.[22] BP binds to guinea pig lymphocytes with cytophilic antibody.[23] All these studies were done with whole BP. Our inhibition studies provide evidence of an antigen-specific receptor on T cells for the disease-inducing determinant.

The percent of antigen-binding cells noted in the present study is 3- to 10-fold higher than those reported by some,[18,22] but similar to those of others.[17,24,25] Our technique differed significantly from those of Ortiz-Ortiz and Weigle[18] and Yung and associates.[22] Our experiments were conducted at 37° C rather than 4° C, binding was studied without azide, and antigen concentrations differed. Hammerling and McDevitt[27] and we have shown that optimal binding to T cells is an energy-dependent process, inhibited by cold and metabolic uncouplers.[24-26]

In these experiments there was no clinical evidence of EAE in recipient guinea pigs, even though sensitization against the major encephalitogenic determinant was demonstrated. In previous studies, 20-fold higher doses of in vitro-sensitized thymic lymphocytes were required to elicit EAE.[28] Thus, although the conditions used in our experiments were inadequate to induce EAE, the results were compatible with the notion that EP receptor-bearing T cells may be triggered to immune differentiation when EP is associated with syngeneic macrophages.

How the mode of presentation of BP determines whether sensitized RTLs are recruited is an open question. Two possibilities are attractive. First, soluble BP may directly prevent the activation of ITL sensitive to BP macrophages by blockade of BP receptors on the ITL. T-cell recognition of antigen associated with macrophage involves a dual recognition of both the antigen and a portion of the major histocompatibility complex associated with immune-response gene products.[29,30] Soluble BP may interfere with this dual recognition. Second, soluble BP may activate a suppressor cell that inhibits RTL. Binding of antigen to different subclasses of T cells is under the control of soluble mediators.[17] Macrophage-derived mediators lead to binding of antigen to helper T cells, whereas soluble antigen naturally binds to suppressor T cells. Whether this phenomenon accounts for our observation is not proved. Nevertheless, soluble central nervous system antigen may play a physiologic role in maintaining tolerance.[31] Elucidation of such putative mechanisms in experimental autoimmune diseases may have direct application to the recent therapeutic endeavors in which soluble BP has been used in attempts to suppress multiple sclerosis.[32-34]

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