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EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE) MEDIATED BY T CELL LINES: PROCESS OF SELECTION OF LINES AND CHARACTERIZATION OF THE CELLS[1]

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This paper describes alternative ways of deriving lines of rat lymphocytes capable of mediating EAE. The cells were demonstrated to bear markers specific for T lymphocytes and to be negative for surface Ig, and for Fc and complement receptors. The line cells were found to proliferate *in vitro* specifically in response to myelin basic protein (BP) associated with accessory cells syngeneic at the major histocompatibility complex (MHC). This accessory cell function could not be provided by purified populations of adherent cells from spleen, peritoneal exudates, or bone marrow cultures alone.

Experimental autoimmune encephalomyelitis (EAE)[2] is a T cell-mediated autoimmune neurologic disease characterized by clinical paralysis and mononuclear cell inflammatory lesions in the central nervous system. EAE can be induced in genetically susceptible animals by actively immunizing them against myelin basic protein (BP) (1). To investigate the pathophysiology and immune regulation of EAE we have developed stable lines of rat T lymphocytes specifically reactive against BP (2, 3). Intravenous inoculation of relatively small numbers of cells of these lines into naive syngeneic recipient rats produced EAE within a few days. Furthermore, these anti-BP T cell lines could be used to vaccinate rats against EAE. A single i.v. inoculation of line cells attenuated by irradiation (1500 R) or treatment with mitomycin C produced resistance to subsequent EAE induced by active immunization against BP in about 65% of recipients (4). Thus, the anti-BP cell lines are functional in mediating or protecting against EAE.

In this paper, we describe alternate methods for selecting and maintaining functional lines of anti-BP cells, and characterize their immunospecificity, their surface markers, and the nature of the accessory cells required for the proliferative response of the line cells to BP.

MATERIALS AND METHODS

Rats. Inbred Lewis (RT1-l), BN (RT1-n) and (Lewis × BN)F_1 hybrid rats and L.BN (RT1-n) congenic rats were supplied by the Animal Breeding Center of the Weizmann Institute of Science. Animals were used between 2 and 4 mo of age and were matched for age and sex in each experiment.

Antigens. BP was extracted from guinea pig spinal cords as described by Hirshfeld *et al.* (5), but was not purified by column chromatography. The purified protein derivative (PPD) of tuberculin was purchased from Statens Serum Institute, Copenhagen, Denmark; and concanavalin A (Con A) from Miles-Yeda, Rehovot, Israel.

Immunization of animals. EAE was induced in Lewis rats by injecting each hind foot pad with 0.05 ml of an emulsion of 25 μg BP in phosphate-buffered saline (PBS) emulsified with an equal volume of Freund's adjuvant (BP/CFA) containing 200 μg *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). For selection and separation of lymphoblasts to specific antigens, animals were injected with BP/CFA in all four footpads, 0.05 ml in each.

Antibodies and antisera. Monoclonal antibodies, the MAS-010 specific for all rat T lymphocytes, and MAS-011, shown to be specific for a subclass of T lymphocytes with helper function (6), were obtained from Sera-Lab, England. Fluoresceinated rabbit anti-rat Ig antisera (16 mg/ml) were obtained from Miles-Yeda, Rohovot. The rabbit anti-rat Ig was absorbed on Sephadex-mouse Ig immunoabsorbent before fluoresceination. Rabbit anti-sheep red blood cells (anti-SRBC) were a gift of Dr. H. Wekerle of the Max-Planck Institut für Immunbiologie, Freiburg, Federal Republic of Germany.

Culture medium (2). All cell cultures utilized Dulbecco's modification of Eagle's medium (Grand Island Biological Co.). Medium used for proliferation assay and for selection cultures (proliferation medium) was supplemented with 1 mM glutamine (Bio-Lab, Jerusalem, Israel), 2-mercaptoethanol (5×10^{-5} M), gentamicin (40 μg/ml), and 1% fresh autologous rat serum. The medium used to maintain and propagate antigen-specific cell lines in long-term culture (propagation medium) was the proliferation medium supplemented with 15% (v/v) of supernatant of Con A-stimulated lymphocytes as a source for T cell growth factor (TCGF) prepared as described below, 10% horse serum (GIBCO), 1 mM sodium pyruvate, and nonessential amino acids (Bio-Lab).

In vitro proliferative response of lymph node cells (LNC) (2). Draining lymph nodes of immunized Lewis rats were removed and pooled in ice-cold PBS. A single cell suspension was prepared, and the cells were seeded in flat-bottom microtiter plates (Falcon 3040) in quadruplicate wells. 1×10^6 LNC were cultured in each well in 0.2 ml of culture medium (proliferation medium) with various antigens in the following concentrations: 50 μg/ml BP, 25 μg/ml PPD, 5 μg/ml Con A, or as otherwise indicated. The cultures were incubated for 72 hr at 37°C in humidified air plus 7.5% CO_2. The cultures were pulsed with 1 μCi of ^{3}H-thymidine (specific activity 10 Ci/mmol; Nuclear Research Center, Negev, Israel; Ci = 3.7×10^{10} bequerels) for the last 18 hr of incubation. The cultures were harvested on fiberglass filters by a multiharvester, and incorporation of thymidine was measured in a liquid scintillation counter. The proliferative response was expressed as Δ cpm (mean cpm in test cultures minus mean cpm in control cultures without antigen) or as otherwise stated.

In vitro proliferative response of specific lymphoblasts and line cells (2). The standard assay of the proliferative response of cell lines or of antigen-selected lymphoblasts to the specific antigens was assayed in quadruplicate wells in flat-bottom microtiter plates (Falcon 3040). Each well contained 2.5×10^4 specific lymphoblasts, 1×10^6 syngeneic accessory cells in the form of irradiated normal LNC (irradiation conditions: 1500 R from a ^{60}Co gamma source, from a distance of 40 cm, dose rate 450 rad/min; Atomic Energy of Canada, Ottawa), and antigen, as described above, in 0.2 ml of proliferation medium. After 24 hr of incubation, the cultures were pulsed with ^{3}H-thymidine for another 18 hr and then harvested, and the proliferative response was measured as described above. In some experiments, we studied the kinetics or dose response of line cells; in the proliferation assay, we used various numbers of specific cells and different times of incubation, as indicated. In the dose-response experiment that lasted 72 hr we used round-bottom microtiter plates (Nunc, Denmark). Each well contained various numbers of line cells, 0.5×10^6 irradiated accessory lymph node cells, and antigens in 0.2 ml, as described above. The cultures were pulsed with ^{3}H-thymidine (1 μCi) for the last 18 hr, as above.

Separation of antigen-specific lymphoblasts using a discontinuous Ficoll density gradient was carried out as previously described (2). This procedure for generation of specific lymphoblasts has a relatively high yield. Out of 45×10^7 LNC cultured for 72 hr with PPD or BP, about 60% of the seeded cells were recovered as viable cells. About 20 to 30% of them could be defined microscopically as large lymphocytes or lymphoblasts. After sepa-

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[1] This work was supported by grants from the Stiftung Volkswagenwerk, Hanover, Federal Republic of Germany, and by Grant NS 18168 from the National Institutes of Health, U. S. A.
[2] Abbreviations used in this paper: BP, myelin basic protein; EA, erythrocytes coated with antibodies; EAC, erythrocytes coated with antibodies and complement; EAE, experimental autoimmune encephalomyelitis; LNC, lymph node cells; MHC, major histocompatibility complex; TCGF, T cell growth factor (interleukin 2); FACS, fluorescence-activated cell sorter.

ration by centrifugation on the discontinuous Ficoll density gradient, about 40×10^6 lymphoblasts were usually recovered from selection cultures to BP, and 70×10^6 from selection cultures to PPD. Thus, we recovered as pure populations of lymphoblasts about 15% of the starting lymphocyte population, or 30 to 40% of the lymphoblasts applied to the gradient. The isolated antigen-specific lymphoblasts recovered after density separation were continuously propagated (Z series), submitted to experimental protocols, or cryopreserved.

Preparation of TCGF. We used supernatants of Con A-stimulated mouse lymphocytes as a source of TCGF (7). (BALB/c × C57BL/6)F_1 spleen cells were suspended in RPMI 1640 culture medium (10^7/ml) supplemented with 1 mM glutamine, 2-mercaptoethanol (5×10^{-5} M), gentamicin (40 μg/ml), 1% fresh autologous mouse serum, and Con A (2.5 μg/ml) and incubated in a humidified incubator with a constant gas flow of 10% CO_2 in air. After 24 hr, the cultures were centrifuged at 900 × G for 10 min, and the supernatant was harvested and stored at −20°C until used.

In vitro selection and isolation of antigen-specific lymphoblasts. Lines were developed from antigen-specific T lymphoblasts that were separated using a density gradient (the Z line series) or were not separated (the U line series). A single cell suspension was prepared from pooled lymph nodes removed from Lewis rats injected 9 days earlier with BP/CFA in four footpads. The cells were resuspended in proliferation culture medium (5×10^6/ml) and plated in 60-mm petri dishes (6 ml/dish; Nunc, Denmark). To select lymphoblasts specific to PPD or BP, some cell suspensions were cultured with PPD (30 μg/ml) and some with BP (60 μg/ml). After 72 hr of incubation, the cells were collected and washed three times by mild centrifugation using a table centrifuge. The cells were then submitted either to a procedure of separation of lymphoblasts from small cells by using a discontinuous Ficoll density gradient (2) or to a further selection procedure as follows.

Cells undergoing further selection were resuspended in propagation culture medium (2×10^5/ml) and reseeded in large petri dishes (10 ml/dish; Nunc, Denmark). The cultures were incubated for 24 to 48 hr at 37°C in a humidified atmosphere plus 7.5% CO_2. The cells were then collected, washed three times by centrifugation, and resuspended in proliferation culture medium (2×10^5/ml) together with irradiated (1500 R) syngeneic accessory LNC (10×10^6/ml) and PPD (30 μg/ml) or BP (60 μg/ml) correspondingly in 100-mm petri dishes (10 ml/dish). The cultures were incubated for 72 hr at 37°C in a humidified atmosphere plus 7.5% CO_2, and the cells were collected, washed three times by mild centrifugation, and were either continuously propagated (U series) and used in experimental protocols, or cryopreserved.

Propagation of lines of lymphoblasts. Selected lymphoblasts were grown continuously in propagation medium. The cells were grown in 100-mm plastic tissue culture dishes at an initial concentration of 2×10^5/ml of propagation medium in the absence of antigen or accessory cells. The cultures were transferred every 3 to 4 days and maintained *in vitro* for months.

Cryopreservation of specific lymphoblasts. Specific lymphoblasts that were selected or propagated as a continuous line were sedimented by centrifugation and resuspended in propagation medium (10^7 cells/ml). An equal volume of ice-cold medium containing 20% dimethyl sulfoxide (DMSO) was slowly added. The mixtures were stored in styropore containers and frozen overnight at −80°C and subsequently kept in liquid nitrogen.

Mixed lymphocyte culture (MLC). Single cell suspensions of irradiated (1500 R) stimulator mesenteric LNC of normal Lewis and BN rats, and of responder normal Lewis mesenteric LNC or draining LNC of Lewis rats primed 9 days earlier with BP/CFA, were resuspended (5×10^6/ml) in proliferation medium. Line cells were used at a concentration of 5×10^5/ml in proliferation medium. Aliquots of 0.1 ml of stimulator cells and of 0.1 ml of responder cells were seeded in each culture well of flat-bottom microtiter plates. The cultures were incubated for 86 hr at 37°C in humidified air plus 7.5% CO_2, with ^{3}H-thymidine (1 μCi) for the last 18 hr. The cultures were harvested as described above, and the proliferative response of responder cells was expressed as the mean cpm of quadruplicates.

Analysis of membrane markers of line cells. Fluorescence analysis of specific rat T lymphocyte markers with monoclonal antibodies was done using the fluorescence-activated cell sorter (FACS II; Becton-Dickinson Electronics Lab., Mountain View, CA). Viable line cells (2×10^6) or normal thymus cells in 0.1 ml of PBS were incubated for 30 min at 4°C with 10 μl of MAS-010 or MAS-011 monoclonal antibodies at the appropriate dilutions chosen for staining. The cells were then washed twice and incubated for 30 min at 4°C, with 10 μl of fluoresceinated rabbit anti-rat Ig. After further washings, the cells were resuspended in 2 ml of 0.02% sodium azide-PBS, kept in ice, and analyzed for their relative scattering and fluorescence using the FACS II. The frequency and fluorescence profile of the stained cells were determined by exciting the cells with a 488-nm line operated at 400 meV laser light output. The photomultiplier was set at 550 V, and the fluorescent signals were detected with an S-11 photomultiplier tube. At least 10,000 viable cells (as gated by scatter analysis) were analyzed for fluorescence intensity at different gains.

Fc and complement (C) receptors on line cells were analyzed using a slightly modified version of the technique described by Parish and Hayward (8). Briefly, SRBC were packed and suspended in PBS to give a concentration of 10% (v/v). Five milliliters of 10% SRBC were added with 5 ml of diluted rabbit anti-SRBC antiserum (1:125, determined as subagglutinating titer) and incubated for 20 min at 37°C. The antibody-treated SRBC (EA) were washed twice and resuspended in 5 ml of PBS. Five milliliters of a 1:10 dilution of fresh mouse serum (as a source of C) were added and incubated for 20 min at 37°C. The SRBC treated with antibody and C (EAC) were washed twice and reconcentrated to 20% (v/v) of packed SRBC. A suspension of lymphocytes (2.5 ml; 4×10^7 cells/ml in medium plus 5% fetal calf serum) was mixed with an equal volume of EAC (20%) and incubated for 15 min in a roller tube at 37°C. The suspension was pipetted over a Ficoll/Hypaque layer and centrifuged at 3000 × G for 30 min at room temperature. The erythrocytes of the sedimented fraction were lysed by 0.17 M NH_4Cl treatment at ice temperature. The nonrosetting lymphocytes contained virtually all the cells responsive to Con A and less than 5% lymphocytes expressing membrane Ig as detected by immunofluorescence. The rosetting fraction contained more than 80% Fc-positive cells.

Transfer of EAE by T cell lines specific to BP. The U and Z series of lines that were continuously propagated *in vitro* in the absence of antigen or accessory cells were transferred into normal syngeneic recipients. Before transfer, each cell line (2×10^5/ml) was incubated with its specific antigen, BP (10 μg/ml) or PPD (5 μg/ml), in the presence of syngeneic irradiated (1500 R) thymus cells (10×10^6/ml). After 72 hr of incubation, the cells were collected, then washed twice, and various concentrations of lymphoblasts in a volume of 1 ml of PBS were injected into the tail vein of female Lewis rats. Among the injected cells, at least 85% were viable lymphoblasts. The vast majority of accessory cells apparently had died and disintegrated. The recipient rats were observed daily for the development of EAE. Minimal clinical signs of EAE were considered to be frank paralysis of the hind limbs. Histologic evidence of EAE consisted of perivascular infiltrates of mononuclear cells in the white matter of the central nervous system (2).

RESULTS

Selection, propagation, and the proliferative response of T cell lines reactive against BP. Rats were immunized with BP or other antigens emulsified in CFA, and 9 days later cells obtained from the draining lymph nodes were incubated with the selecting antigen for 3 days *in vitro*. We have previously described (2) the technique of separating lymphoblasts using a Ficoll density gradient and propagating the lymphoblasts as a line using TCGF. Lines obtained in this manner were termed the Z series lines. The U series of lines were obtained by replacing density separation of the lymphoblasts by 1 to 2 days of growth in medium containing TCGF and a second 3-day selection with specific antigen. Figure 1*A* shows the immunospecificity of unselected populations of LNC from rats primed with BP/CFA, and Figure 1*B* shows the immunospecificities of selected lymphocytes, unseparated or separated by a density gradient. It can be seen (Fig. 1*A*) that the primed LNC responded to BP, PPD, and the mitogen Con A, whereas normal, unprimed LNC responded only to Con A. In contrast (Fig.1*B*), after selection of cells that responded either to BP (*a*) or to PPD (*b*), the cells responded to the selecting antigen but not to the unselecting antigen. All cells responded to Con A. Note that the response to the accompanying antigen was lost whether or not the lymphoblasts were separated by the density gradient. Hence, incubation with the selecting antigen (BP or PPD) was sufficient to extinguish reactivity against the unselecting antigen even though reactivity to both BP and PPD was present in the initial BP/CFA-primed population of LNC.

The antigen-specific lymphoblasts that were obtained by *in vitro* selection with or without separation by density gradient were propagated *in vitro* as continuous lines by culturing them in a propagation culture medium containing TCGF in the absence of antigen or accessory cells. Restimulation of lines for 3 days with specific antigen together with accessory cells was carried out at monthly intervals for the first 2 mo of growth; subsequently, the lines were maintained without antigen or accessory cells for about 9 mo (U series) or for over 18 mo (Z series). If we assume that all the cells replicate, the generation time of our lines is about 24 to 30 hr. Figure 2 shows that

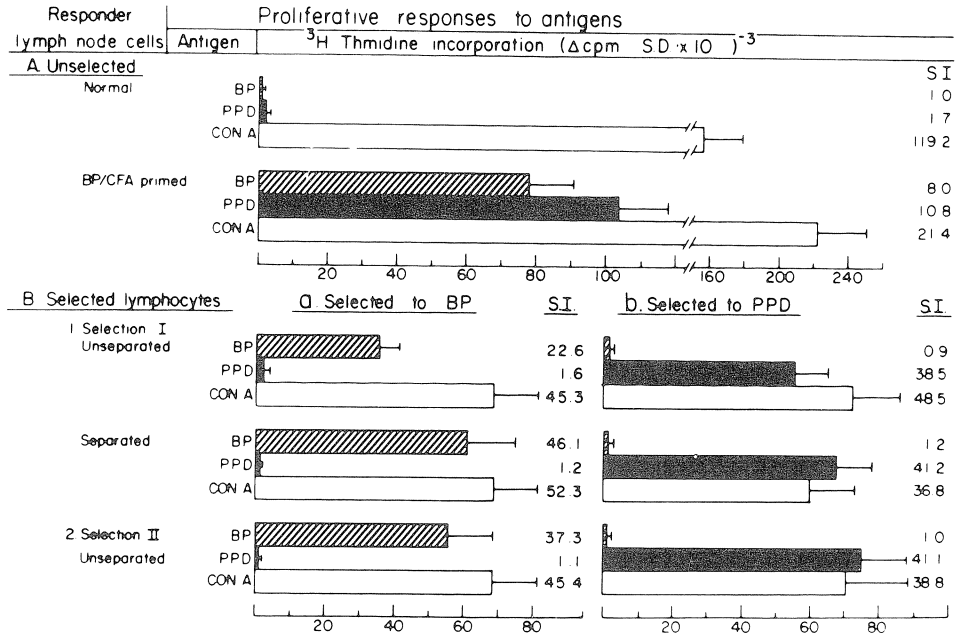

Figure 1. Antigen specificity of BP/CFA-primed LNC and antigen-selected lymphoblasts. On day 9 after immunization with BP/CFA, draining LNC were assayed for their proliferative response to antigens, either directly (*A. Unselected*) or following incubation for 72 hr (*B. Selected*) with antigens BP (B-1a) or PPD (B-1b). Part of the cells obtained after this first period of selection were separated on a Ficoll density gradient, assayed for their proliferative response, and then maintained in propagation medium as Z lines. These cells are referred to as Separated. Another part of the cells selected in B-1 were not separated by density, but were grown in propagation medium for 48 hr and then restimulated with specific antigen, either BP or PPD for a second period of selection of 72 hr (Selection II). These unseparated cells selected to BP (B-2a) or to PPD (B-2b) were assayed for their antigen specificity and were then maintained in propagation medium as U lines.

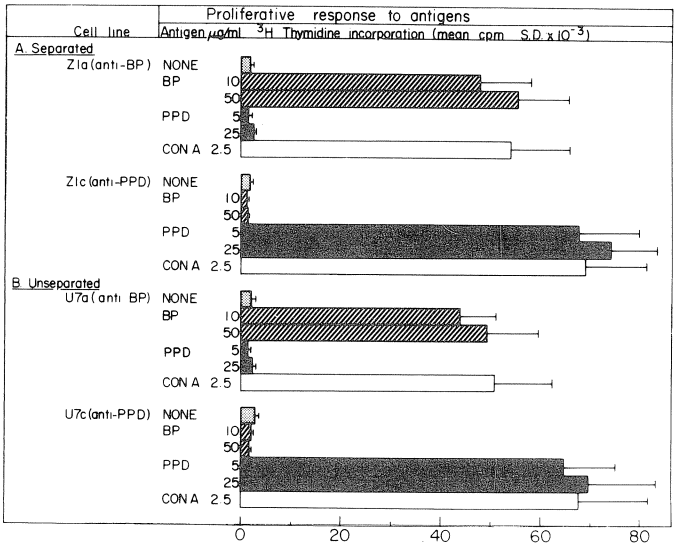

Figure 2. T cell lines retain their antigen specificity after long-term *in vitro* propagation. Lymphoblasts of T cell lines that originated from antigen-selected and separated (*A, the Z series*) or unseparated (*B, the U series*) were assayed for their response to BP, PPD, or Con A. Z lines were propagated *in vitro* for over 18 mo and U lines for about 8 mo before assay.

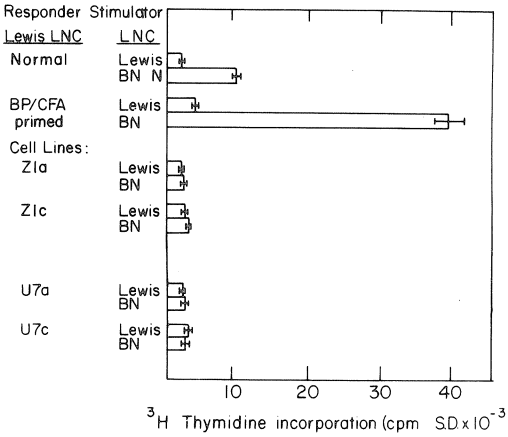

Figure 3. Antigen-selected T cell lines are depleted of alloreactive cells. Lewis lymph node lymphocytes were assayed for their reactivities to irradiated allogeneic BN stimulator lymph node cells before or after inoculation with BP/CFA, and after their selection as cell lines.

immunospecificity was preserved both in lines derived from density gradient separated lymphoblasts, the Z1a and Z1c lines, and in unseparated lines, the U7a and U7b lines. Once a line was established, reactivity to the accompanying, unselecting antigen could not be regained by culture with that antigen. An anti-BP line remained nonreactive to PPD despite repeated culture of the cells with PPD (data not shown). Hence, immunospecificity remained stable.

In addition to a lack of response to the unselecting antigen, the lines also lost their reactivity to allogeneic antigens in the MLC (Fig. 3). LNC from Lewis rats that had been immunized with BP/CFA showed a stronger MLC to allogeneic BN stimulator cells than normal Lewis lymph node cells did. However, the anti-BP and anti-PPD lines that were derived from the primed LNC had no reactivity against allogeneic BN rat stimulator cells. These results indicate that the proliferative responses of the cell lines were restricted to the selecting antigen.

Experiments were done to investigate the number of line cells and the times of incubation with antigen needed for an optimum proliferative response. In proliferative assays of 72 hr, as few as 100 cells per well produced a significant specific anti-BP response. Higher numbers of cells showed much more active responses. In assays of 40 hr, responses were also high and there were relatively lower background cpm for a given number of cells than were obtained in 72-hr assays. Hence, we elected to use 2.5×10^4 cells in a 40-hr assay as our standard procedure for measuring antigen-specific proliferative responses.

Line cells are Fc$^-$, C$^-$ T lymphocytes. Experiments were done to study the surface markers of the line cells. Figure 4 shows the binding, using a FACS II, of two types of monoclonal antibodies specific for rat T lymphocytes to normal rat thymus and Z1a line cells. MAS-010 was shown to be a marker on all T lymphocytes and MAS-011 on rat T lymphocytes with helper function (6). We found that almost 100% of the cells were strongly stained by both antibodies (Fig. 4). However, the staining with the MAS-010 antibodies appeared to be stronger than that obtained with the MAS-011 antibodies. This indicates that the line cells were T lymphocytes. At present, it is not

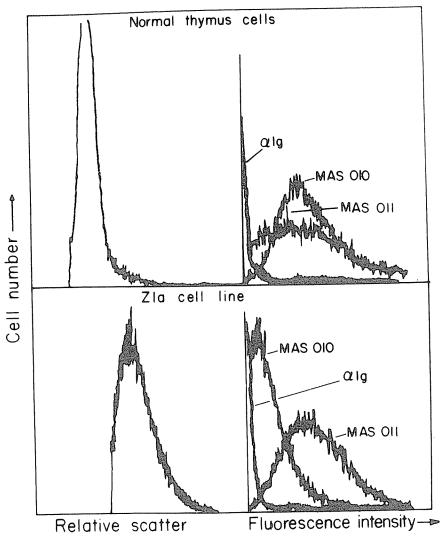

Figure 4. Binding of monoclonal antibodies specific to rat T lymphocytes. Scatter and fluorescence distribution profiles of normal thymus and Z1a line cells that were stained with monoclonal antibodies specific to all rat T lymphocytes (MAS-010), with monoclonal antibodies specific to a class of T lymphocytes with helper function (MAS-011) or with rabbit anti-rat-Ig antiserum.

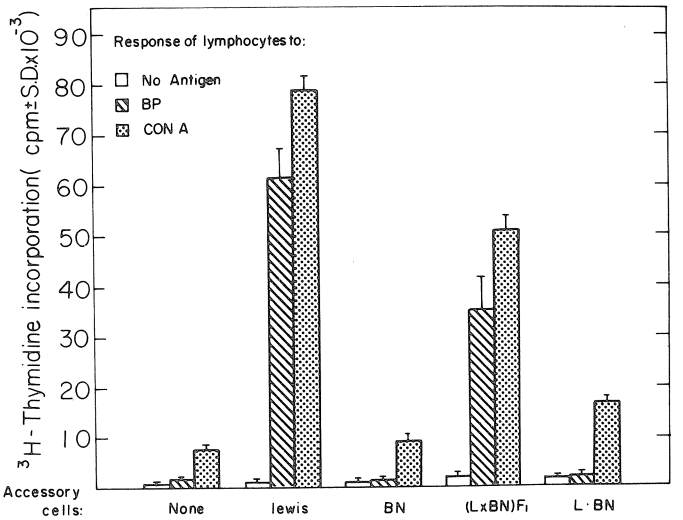

Figure 5. Z1a T cell line recognizes BP in association with MHC gene products. Lymphoblasts of Z1a line were assayed for their response to BP or Con A in the presence or absence of irradiated lymph node accessory cells originating from syngeneic Lewis, RT1 congenic L.BN, allogeneic BN, or semi-syngeneic (L × BN)F_1 rats.

TABLE I

Z1a line lymphoblasts are negative for Fc and C receptors[a]

Fractionation of Selected Lymphoblasts	Proliferative Response to Antigens (cpm × 10^{-3})			
	No antigen	BP	Con A	PHA
A. Nonfractionated	1.7 ± 0.3	57.5 ± 6	72.1 ± 3	46.3 ± 7
B. EA fractionation:				
Fc^+	1.2 ± 0.4	3.6 ± 0.9	15.1 ± 2	7.9 ± 2
Fc^-	2.6 ± 0.4	42.7 ± 7	78.0 ± 7	38.5 ± 5
C. EAC fractionation				
C^+	2.1 ± 0.4	4.7 ± 0.8	9.4 ± 1	6.4 ± 1
C^-	1.8 ± 0.3	53.1 ± 6	69.8 ± 8	40.4 ± 3

[a] Z1a line cells were fractionated by EAE- and EAC-rosette techniques. The 5% of cells that were recovered as Fc^+ or C^+ were concentrated and they and the Fc^- and C^- cells were assayed for their proliferative responses to BP, Con A, or PHA in the presence of accessory cells.

known whether the MAS-011 marker is restricted to helper T lymphocytes, or whether it is also present on delayed hypersensitivty or cytotoxic T lymphocytes. Therefore, we cannot assign the lines to any particular subclass of T cells. None of the cells of different lines bound anti-rat Ig antisera, including the Z1a line cells (Fig. 4). Table I illustrates that the line cells were negative for Fc or C receptors. Hence, we may conclude that the line cells are Fc^-, C^- T lymphocytes.

Characterization and tissue distribution of accessory cells required for antigen-specific stimulation of line cells. The proliferative responses of the antigen-specific selected lymphoblasts (Fig. 1*B*) and line cells (Fig. 2) to the specific antigens were obtained in the presence of irradiated syngeneic accessory LNC. Figure 5 shows that Z1a line cells could not respond to BP in the absence of accessory cells. Z1c line cells and other T cell lines or unpropagated antigen-selected lymphoblasts that we have obtained required syngeneic accessory cells for their proliferative responses to antigens (data not shown).

Furthermore, Figure 5 also shows that the proliferative response of line cells to specific antigen was dependent on RT1 syngeneic irradiated accessory cells. Allogeneic BN or congenic L.BN cells that differed from the Lewis line cells only at the RT1 complex failed to support the response of Z1a line cells against BP. (Lewis × BN)F_1 accessory cells produced a somewhat decreased response, indicating a possible gene-dose effect in accessory function.

TABLE II

Response of selected specific T lymphoblasts to antigen-pulsed accessory cells

Lymphoblasts[a] Specific to:	Accessory Cells[b]	Antigen-Pulsed	Proliferative Responses (Mean Cpm ± SD × 10^{-3})	Δcpm
BP (Z1a line)	Lymph node	−	1.9 ± 0.3	36,482
		+	38.3 ± 4.1	
	Spleen	−	0.8 ± 0.1	41,878
		+	42.7 ± 6.0	
PPD (Z1c line)	Lymph node	−	1.4 ± 2.1	70,111
		+	71.5 ± 10.2	
	Spleen	−	0.9 ± 0.1	69,410
		+	70.3 ± 8.6	

[a] 2.5 × 10^4 lymphoblasts of Z1a or Z1c were cultured for 40 hr with the relevant antigen-pulsed accessory cells and their proliferation response was measured.

[b] Accessory cells were suspensions of syngeneic Lewis lymph node or spleen cells. The cells were irradiated (1500 R) and some were pulsed with either BP or PPD, by incubating them with 100 μg/10^7 cells/ml for 45 min. The accessory cells were washed and 10^6 unpulsed or pulsed with the specific selected antigen were added to each lymphoblast cultured well.

Table II tabulates the positive responses of the Z1a anti-BP and Z1c anti-PPD lines to antigen-pulsed syngeneic lymph node or spleen accessory cells. These results indicate that accessory cells can function to present antigen to the line cells.

We attempted to further define the accessory cells by testing the function of accessory cells obtained from various organs. Table III shows that lymph node, thymus, and spleen cells provided accessory function. It was interesting, however, to find that a variety of purified populations of macrophages could not act as accessory cells for lines, although such cells were found to be capable of presenting antigen to primed LNC (not shown). Peritoneal exudate cells, stimulated or not by injection of proteose peptone broth, cultures of bone marrow macrophages, and the adherent cells of the spleen all failed to provide accessory function. This suggests that the line cells may need the help of another class of cells in addition to macrophages.

Mediation of EAE. Experiments were done to compare the effectiveness of Z1a cell lines propagated for over 18 mo and U7a cell lines propagated for about 8 mo in mediating EAE. Lewis rats were injected i.v. with various numbers of cells of the Z1a or the U7a T lymphocyte lines selected for their

TABLE III

Tissue distribution of accessory cells

Origin of Accessory Cells	Proliferative Response to Antigens (cpm ± SD × 10^{-3})		
	No antigen	BP	Con A
Lymph node	1,641 ± 126	74,369± 5,811	59,740 ± 4,217
Spleen	1,041 ± 99	30,299± 2,171	41,526 ± 5,993
Thymus	969 ± 102	76,113± 5,513	71,841 ± 6,848
Peritoneal exudate cells:			
Normal washout*	215 ± 18	372± 63	2,117 ± 122
Protease/peptone stimulated*	391 ± 66	422± 81	1,642 ± 195
Bone marrow macrophages*	244 ± 39	261± 59	1,074 ± 214
Spleen adherent cells*	417 ± 86	614± 114	1,989 ± 178

* 2.5×10^4 Z1a line cells were incubated in round-bottomed microtiter wells with or without antigen, in the presence of irradiated lymph node (5×10^5/culture), spleen (5×10^5/culture), or thymus (1×10^6/culture) accessory cells, or in the presence of normal washout macrophages (1.25×10^3), protease-peptone-stimulated macrophages (1.25×10^3/culture), bone marrow macrophages (1.25×10^3 culture), or spleen nonadherent cells (1.25×10^3/culture). Results obtained by using higher or lower ratios between accessory and line cells were essentially the same. Cells of these origins were tested before for their capability to stimulate BP/CFA primed LNC.

TABLE IV

Intravenous injection of BP specific T lymphoblast lines produces EAE[a]

Cell Line	Antigen Specificity	No. of Cells (1×10^{-6}) Injected I.V.	Clinical EAE		
			Incidence	Mean day of onset	Mean day of recovery
Z1a	BP	20	9/10	2	7
		10	13/15	3	6
		1	21/25	4	6
		0.1	4/5	5	7
		0.01	0/5		
Z1c	PPD	50	0/5		
U7a	BP	20	8/10	3	6
		10	9/10	3	6
		1	7/10	4	6
		0.1	0/10		
U7c	PPD	50	0/5		

[a] Line cells of Z or U series that were propagated for over 18 mo (Z) or 8 mo (U) were restimulated (2×10^5/ml) with BP (10 μg/ml) or with PPD (10 μg/ml) in the presence of syngeneic-irradiated thymus cells (15×10^6/ml) for 72 hr and various numbers of lymphoblast cells were intravenously injected into normal Lewis rats. The animals were observed for the appearance of overt paralysis as a sign of EAE.

response to BP, or with the Z1c or the U7c lines selected for their response to PPD. Table IV illustrates that both the Z1a and the U7a anti-BP lines, but not the anti-PPD lines, could mediate EAE upon i.v. inoculation into naive recipient rats. The dose-response characteristics of the Z1a line suggested that it was somewhat more efficient than the U7a line that had been selected without the step of density gradient separation. The Z1a line mediated EAE upon injection of as few as 10^5 cells, whereas 10^6 cells or more of the U7a line were required to produce disease.

DISCUSSION

We have previously demonstrated that anti-BP T cell lines can produce EAE in naive recipients (2), and that these cells when attenuated could be used to induce resistance to EAE (4). This paper relates to the selection and characterization of these stable functional T cell lines.

We found that such lines were obtainable either with or without the procedure of density separation of lymphoblasts. Cells of the separated Z1a line and of the selected but unseparated U7a line demonstrated strict antigen specificity for BP, both before (Fig. 1) and after propagation as lines (Fig. 2). In addition to losing responsiveness to the PPD antigen that was present in the original populations of primed LNC (Fig. 1), both types of lines lost reactivity to allogeneic antigens in an MLC assay (Fig. 3). However, the Z1a line seemed to be active in mediating EAE at a lower cell number than the U7a line (Table IV). This suggests that the Z1a line was richer in the numbers or the specific activity of EAE effector cells.

The reasons for differences in degree of activity are unknown, and it remains to be seen whether they could be related to the different methods of selection. The Z1a line has been remarkably stable during cultivation *in vitro* for over a year and a half, whereas the U7a line has been maintained for only about 9 mo. Therefore, we do not know whether the U7a line will prove to be as stable. Thus far, however, we have observed no differences between the lines other than those just described.

Analysis of the surface markers of the lines indicates that they are Fc^-, C^- T lymphocytes (Fig. 4 and Table I). The finding that these lines of T lymphocytes mediate EAE contributes to the understanding of the pathophysiology of EAE. They provide direct evidence that T lymphocytes specifically reactive against BP can mediate EAE. However, in the absence of functional markers for rat subclasses of T lymphocytes, it is difficult to identify the subclasses of T cells in the lines. The MAS-011 monoclonal antibodies were found to bind to rat T lymphocytes with helper function, but were not shown to be restricted to such cells (6). Therefore, we cannot exclude the possibility that the line cells include delayed hypersensitivity or cytotoxic types of lymphocytes, as well as helper T lymphocytes.

The T cell lines responded to their specific antigens by proliferating *in vitro* only in the presence of accessory cells syngeneic at the major histocompatibility complex (MHC) (Fig. 5), as has been demonstrated in other systems (9, 10). These accessory cells appeared to present specific antigen to the T cells (Table II), suggesting that they could be radioresistant antigen-presenting cells belonging to the family of macrophages (11) or dendritic cells (12). Populations of cells from the lymph nodes, thymus, and spleen, all containing macrophages (13), functioned as accessory cells (Table III). However, enriched populations of macrophages including peritoneal exudate cells (14), adherent spleen cells (15), or bone marrow cultures (16) failed to provide accessory cell function to the T lymphocyte lines (Table III), although these populations of cells have been shown to be capable of presenting specific antigens to *in vivo* primed T lymphocytes (14–16). It is conceivable that antigen presentation to T lymphocytes involves activation of the accessory cells, possibly by an interleukin secreted by an additional cell type. The cultures of selected T lymphocyte lines may become depleted of these additional cells, so that the lines cannot respond in the presence of purified cultures of BP-pulsed macrophages or reticulum cells alone.

The anti-BP lines used in this study were not cloned, and thus the population of cells mediating EAE may have included a number of BP-sensitive clones, not all of which were responsible for EAE. However, we recently cloned the Z1a line and found that a single anti-BP clone was capable of mediating EAE (manuscript in preparation). Because BP was the only antigen to which the EAE effector cells responded, it would appear that these cells are able to recognize BP as it exists *in vivo* in normal rats. We have found (manuscript in preparation) that anti-BP line cells specifically accumulate in the central nervous system when they mediate EAE. Thus, whatever accessory cells are required for this recognition *in vivo* would seem to be available along with BP.

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