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The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis*

The isolation and propagation of functional antigen-specific lines of T lymphoblasts is described. These lines were found to recognize foreign or self antigens in association with accessory cells of syngeneic major histocompatibility complex genotype. Intravenous inoculation of a T cell reactive only against myelin basic protein led to development of clinical paralysis in syngeneic rats. Thus, it is possible to study biological function as well as antigen specificity using T cell lines.

1 Introduction

The nature of the receptor for antigen of T lymphocytes [1, 2], the involvement of gene products of the major histocompatibility complex (MHC) in this receptor, and the mechanism of action of immune response genes are widely studied problems in immunology [3]. Solution of these problems would be facilitated by the availability of pure populations of antigen-specific T lymphocytes in large quantities. Furthermore, such T cell lines might also be useful in investigating the pathophysiology of autoimmune diseases. The recent discovery of T cell growth factors and their effect on the continuous propagation of T lymphocytes has provided a method for developing T lymphocyte lines and clones [4–7] with defined antigen specificity. We report that it is possible to obtain large numbers of clonable T lymphocytes specific for self or non-self antigens, using a one-step technique of separating selected lymphoblasts on a Ficoll density gradient. These lymphocytes respond *in vitro* by proliferating exclusively in the presence of specific antigen together with MHC-restricted antigen-presenting cells. Antigen specificity is retained by these T lymphocytes despite propagation *in vitro* for months. Furthermore, a line of lymphoblasts specifically reactive against myelin basic protein (BP) was found to be functional *in vivo* in mediating experimental autoimmune encephalomyelitis (EAE).

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Abbreviations: MHC: Major histocompatibility complex **BP:** Myelin basic protein **EAE:** Experimental autoimmune encephalomyelitis **CFA:** Complete Freund's adjuvant **OA:** Ovalbumin **PPD:** Purified protein derivative of mycobacteria **SA:** Sheep albumin **Con A:** Concanavalin A **LN:** Lymph node

2 Materials and methods

2.1 Immunization of Lewis rats

Lewis rats were inoculated intradermally with antigens emulsified in complete Freund's adjuvant (CFA). Ovalbumin (OA) was used as a foreign antigen, and BP from guinea pigs was used as an autoimmune antigen. (Injection of BP in CFA induces EAE in Lewis rats [8]).

Three-month-old female Lewis rats were injected into each of the four footpads with 0.05 ml containing either BP (25 μg; extracted from guinea pig spinal cords) or OA (50 μg; Sigma Chemical Co., St. Louis, MO), emulsified in equal volumes of phosphate-buffered saline and CFA containing 4 mg/ml of *M. tuberculosis* H_{37}Ra (Difco, Detroit, MI) (BP/CFA or OA/CFA, respectively). On day 9 after immunization, the draining lymph nodes (LN) were removed from groups of 4 animals each, and suspensions of LN cells were pooled for each group and either assayed directly for their *in vitro* proliferative response to antigens or submitted to the selection and separation of antigen-specific lymphoblasts.

2.2 Isolation of antigen-specific lymphoblasts

LN cells from immunized Lewis rats were suspended (5×10^6 / ml) in Eagle's medium supplemented with 1% fresh autologous serum, 2-mercaptoethanol (5×10^{-5}M) and antibiotics, and cultured in 60-mm petri dishes (6 ml/dish) with added antigens: OA (60 μg/ml; Table 1, group 3), purified protein derivative (PPD; 30 μg/ml; groups 4 and 7) or BP (60 μg/ml; group 6). Following 72 h of incubation at 37 °C in a humidified atmosphere plus 7.5% CO_2, the cells were collected, and the lymphoblasts were separated by centrifugation using a Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient [9]. Cells were resuspended in 1 ml of HEPES-buffered

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Eagle's medium and mixed with 6 ml of Ficoll stock solution (12.8 g Ficoll dissolved in 30 ml medium). The cell suspension was placed at the bottom of plastic tubes fitting a Beckman SW-27 rotor. Six ml of each of decreasing Ficoll densities of 1.08, 1.07, 1.06 and 1.05 g/ml were layered sequentially and overlayered with 4 ml of medium. The gradients were centrifuged at 10000 rpm (about 14000 × g) for 60 min at 4 °C. The fraction containing over 90% lymphoblasts was found in the interface between densities of 1.06 and 1.05 g/ml. This fraction was recovered by pipeting, washed twice by centrifugation, and the cells were cultured *in vitro* in Eagle's medium supplemented with 15% (v/v) of supernatant of concanavalin A (Con A)-stimulated lymphocytes [10], 10% horse serum, 2-mercaptoethanol and antibiotics, without added antigen. Twenty-four hours later, they were submitted to a proliferative assay for studying their antigen specificity.

2.3 Proliferative assays of primed lymphocytes or selected lymphoblasts

LN cells (1×10^6) from primed rats (Table 1; groups 1, 2 and 5) were cultured in quadruplicate in flat-bottom microtiter wells in 0.2 ml of Eagle's medium supplemented with 1% fresh autologous rat serum, 2-mercaptoethanol, antibiotics and antigens in the concentrations indicated below. The proliferative response was determined by measuring the incorporation of [^{3}H]thymidine (1 μCi/well, spec. act. 10 Ci/mmol = 370 GBq/mmol, Nuclear Research Center, Negev, Israel) that was added for the last 16 h of a 72-h culture. The proliferative response of the selected and separated specific lymphoblasts (Table 1; groups 3, 4, 6 and 7 and Tables 2 and 3) were tested using a modified procedure. Cells (2.5×10^4) were cultured for 36 h in each microtiter well with added accessory cells (1×10^6) that were irradiated (1500 rd) normal syngeneic LN cells. The cultures were pulsed with [^{3}H]thymidine ([^{3}H]dThd) for the last 16 h.

The following antigen concentrations were used: OA, 125 μg/ml; SA (sheep albumin, fraction V, Sigma), 125 μg/ml; PPD, 50 μg/ml; Con A (Miles-Yeda, Rehovot, Israel), 2.5 or 5 μg/ml. In the response to alloantigens, 5×10^5 responder cells were cultured with 5×10^5 irradiated (1500 rd) stimulator cells.

2.4 Propagation of selected T lymphoblasts as continuous line

Lymphoblasts were propagated and maintained *in vitro* in Eagle's medium supplemented with 15% (v/v) supernatant of Con A-stimulated spleen cells [10]; 10% horse serum (Gibco, Grand Island, NY); nonessential amino acids (Bio-Lab, Jerusalem, Israel); sodium pyruvate (Bio-Lab), 2-mercaptoethanol and antibiotics (referred to as growth medium). The cells were plated in 100-mm petri dishes (2×10^5/ml) and were transferred every 3–4 days.

2.5 Cloning of T lymphoblasts

Cell lines were obtained from LN lymphoblasts selected either against BP or PPD and propagated *in vitro* for 3 months. The cells were diluted to a concentration of 1 cell/microtiter well in 0.2 ml of growth medium containing 5×10^5 irradiated (1500 rd) syngeneic thymus cells and either BP (10 μg/ml) or PPD (5 μg/ml). Cultures were fed every 2–3 days by replacing 0.1 ml of growth medium supplemented with accessory cells and antigen. After 7 days of culture, the microwells were observed microscopically for proliferation of the cloned lymphoblasts. Positive wells were scored microscopically by the appearance of circumscribed areas of clumps of cells showing lymphoblasts, or by pulsing with [^{3}H]dThd (1 μCi) for the last 16 h of 8 days of culture.

2.6 EAE mediated by T cell line

Lewis rats were inoculated i.v. with various numbers of cells of lines that had been selected for reactivity to BP or PPD and propagated *in vitro* for about 8 months. The rats were observed for the development of overt paralysis of the hind limbs accompanied by histological evidence in the brain of perivascular infiltration of mononuclear cells as minimal signs of EAE.

3 Results

3.1 Antigen specificity of isolated T lymphoblasts

Table 1 shows a comparison of the antigen specificities in a T lymphocyte proliferative response of cells before and after selection. The cells were tested for antigen specificity by incubating them either with the specific antigen used to select the lymphoblasts, or with antigens not used for selection, or with irradiated allogeneic LN cells in the presence of syngeneic accessory cells. LN lymphocytes from uninoculated rats (Table 1, group 1) responded to none of the antigens, except for a response to allogeneic BN LN cells. These lymphocytes could also be stimulated by the mitogen Con A.

LN lymphocytes from rats inoculated with OA in CFA (Table 1, group 2) responded well to OA and showed a cross-reactive response to SA. The population of lymphocytes also responded well to PPD, to allogeneic LN cells and to Con A. However, following selection of lymphoblasts responding to OA (group 3), the selected lymphocytes responded only to OA and to Con A. The population lost the cross-reactive response to SA. This indicates that selection was for clones of lymphocytes responding to immunodominant determinants present on OA, but not on SA. The lymphocyte population also lost the response to PPD and to allogeneic LN cells observed before selection. Note, that the selected lymphocytes showed a high specific response despite the fact that we tested them at a cell concentration 40 times lower (2.5×10^4/well) than that used for nonselected lymphocytes (1×10^6/well) and after incubation for only 36 h compared to 72 h.

When the same population of LN cells was selected for their response to PPD (group 4), upon restimulation the only responses were to PPD and to the Con A mitogen. The clones of lymphocytes that were observed before selection to be responsive to the albumins OA and SA or to allogeneic spleen cells were undetectable after selection to PPD. Populations of LN lymphocytes from rats injected with BP in CFA also lost the reactivities originally present against BP, PPD or allogeneic LN cells, following selection of lymphoblasts responding to one or another antigen; compare groups 5, 6 and 7 in Table 1.

Table 1. Antigen specificity of proliferative response before and after selection of lymphoblasts[a)]

Immunization *in vivo*	Group	Antigen for selection of lymphoblasts	No. of cells × 10^{-4}/well	Proliferative responses (cpm × 10^{-3} ± S.D.)[b)] Soluble antigens					Mixed lymphocyte reaction		Mitogenic response to Con A
				No antigen	OA	SA	PPD	BP	Syngeneic Lewis	Allogeneic BN	
None	1	No selection	100	5 ± 0.5	5 ± 0.4	5 ± 0.2	7 ± 0.5	5 ± 0.5	1 ± 0.1	10 ± 4	189 ± 24
OA/CFA	2	No selection	100	18 ± 14	125 ± 11	72 ± 5	150 ± 14	N.D.	3 ± 1	46 ± 4	248 ± 1
	3	OA	2.5	2 ± 0.2	62 ± 3	3 ± 0.2	3 ± 0.3	N.D.	8 ± 0.6	8 ± 0.7	73 ± 4
	4	PPD	2.5	2 ± 0.2	3 ± 0.2	2 ± 0.1	93 ± 10	N.D.	7 ± 1	7 ± 0.5	110 ± 7
BP/CFA	5	No selection	100	14 ± 2	N.D.	N.D.	130 ± 11	116 ± 9	4 ± 0.8	40 ± 2	322 ± 41
	6	BP	2.5	3 ± 0.2	N.D.	N.D.	4 ± 0.5	68 ± 0.8	6 ± 0.8	6 ± 0.5	92 ± 4
	7	PPD	2.5	2 ± 0.1	N.D.	N.D.	104 ± 7	2 ± 0.2	7 ± 0.4	4 ± 0.4	119 ± 10

a) Lewis rats were injected into the footpads with OA/CFA or BP/CFA. On day 9 after immunization, the draining LN cells were assayed for their *in vitro* proliferative response to antigens either directly (no selection) or after isolation of antigen-specific lymphoblasts on a Ficoll density gradient.

b) Proliferative responses were assayed either after 72 h (groups 1, 2 and 5; no selection) or 36 h (selected groups 3, 4, 6 and 7). Significant responses are underlined. N.D. = not determined.

In addition to a high degree of specificity, this method of separating antigen-specific lymphocytes produces a relatively satisfactory yield. Starting with about 5×10^8 LN lymphocytes, we usually obtain about 3×10^8 lymphocytes after 72 h of culture with a selecting antigen. After separation on the density gradient, we usually harvest about 5×10^7–6×10^7 lymphoblasts. Thus, about 10% of the starting population can be recovered, and this fraction of cells demonstrates a high degree of specificity towards the selecting PPD antigen.

It appeared that the selected lymphoblasts were T lymphocytes, since neither these cells nor their progeny had detectable surface immunoglobulins or receptors for complement or for the Fc fragments of immunoglobulins (results not shown) [11]. Furthermore, they could be stimulated by the T cell mitogens Con A and phytohemagglutinin.

3.2 Isolated specific lymphoblasts recognize the antigen with MHC-syngeneic accessory cells

We found that MHC restrictions were operative between antigen-selected lymphocytes and accessory cells. Table 2 shows the results of an experiment in which lymphocytes selected to BP were activated in the presence or absence of accessory cells of various genotypes. The lymphocytes of Lewis rat origin responded to BP in the presence of syngeneic accessory cells (63×10^3 cpm). They responded less well (36×10^3 cpm) in the presence of accessory cells from (Lewis × BN)F_1 hybrids, and not at all in the presence of allogeneic BN accessory cells (1×10^3 cpm). L.BN rats that differ from Lewis rats only in genes of the MHC also failed to provide accessory cell function for BP-selected lymphocytes of Lewis origin (2×10^3 cpm). Therefore, selected lymphocytes seemed to require MHC-syngeneic accessory cells in addition to specific antigen for their stimulation. This result, too, is compatible with the conclusion that the selected lymphocytes were T lymphocytes [12].

Table 2. Antigen-presenting cells controlled by MHC genes are required for efficient stimulation of selected lymphocytes[a)]

Source of antigen-presenting cells	Response to antigens (cpm ± S.D.) None	BP	Con A
–	722 ± 119	1 859 ± 135	7 518 ± 618
Lewis	1 851 ± 308	62 668 ± 5 091	79 351 ± 3 246
BN	1 064 ± 222	1 167 ± 168	9 488 ± 1 002
(L × BN)F_1	2 112 ± 286	35 842 ± 1 947	51 008 ± 2 762
L.BN	1 781 ± 364	2 065 ± 284	17 646 ± 961

a) Lymphoblasts (2.5×10^4) specific to BP which were selected and separated from BP/CFA-primed LN cells were cultured in quadruplicate in flat-bottom microtiter wells in the presence or absence of irradiated (1500 rd) accessory LN cells of different genotypes (1×10^6/well). The *in vitro* proliferative response to the antigens was assayed for 38 h, as described in Sect. 2.3.

The response of lymphocytes to the mitogen Con A appeared to be relatively less sensitive to the presence of accessory cells than the response to antigen [13, 14]. A moderate but significant response to Con A (7×10^3–8×10^3 cpm) was observed in the absence of accessory cells, or in the presence of MHC-allogeneic accessory cells (Table 2). However, the best response (79×10^3 cpm) was obtained in the presence of syngeneic Lewis accessory cells.

3.3 Propagation of T lymphoblast lines that retain antigen specificity

We have been able for at least 8 months to propagate lymphocytes selected to several specific antigens by culturing the cells in growth medium containing supernatants of lymphocytes

Table 3. Cloning of T lymphoblast lines

Clone	Antigen in cloned well	Microscopic evidence of response[a] (positive/total)	Proliferative response in wells (mean cpm)[b] Positive	Negative	Restimulation of 20 propagated clones (cpm ± SD × 10^{-3}) by antigens[c] None	BP	PPD
Anti-BP	BP	108/384	1651[d]	348	2 ± 1	68 ± 16[d]	2 ± 1
	PPD	0/384	–	682			
Anti-PPD	PPD	123/384	1745[d]	417	1 ± 1	1 ± 1	84 ± 26[d]
	BP	0/384	–	789			

a) Positive wells were scored by the appearance of circumscribed areas of clumps of cells showing lymphoblasts.
b) One 96-microwell plate of each type was pulsed with 1 μCi of [^{3}H]dThd for the last 16 h of 8 days of culture, and the thymidine incorporation in each microwell was measured. The mean cpm of the microscopically observed positive or negative microcultures was calculated.
c) Cloned lymphoblasts of 20 selected wells of each type were expanded *in vitro* by transferring them first into 16-mm tissue culture wells for 4 days and then into 60-mm petri dishes. The cloned cells were expanded in growth medium supplemented with the appropriate antigen and accessory cells (10^6 irradiated thymus cells/ml). After 3 days in 60-mm petri dishes, the cells of each clone were harvested, counted and restimulated *in vitro* by antigens.
d) Statistically significant ($p < 0.001$) by Student's t-test.

Table 4. Anti-BP lymphoblast line mediates EAE[a]

Line	Cells injected i.v. × 10^{-6}	Incidence of EAE	Mean onset of EAE (days)
Anti-BP	1	21/25	3
	25	23/25	2
Anti-PPD	50	0/10	–

a) Male Lewis rats were inoculated with cells from T lymphoblast lines and observed for development of EAE.

stimulated by Con A [4, 5]. This propagation did not require the addition of specific antigen or of accessory cells. However, we found that adding antigen and accessory cells augmented the rate of cell proliferation. Furthermore, these lymphoblasts were less fragile than those propagated with Con A supernatant in the absence of antigen and accessory cells. Immunospecificity was retained after prolonged propagation *in vitro* (not shown) and was stimulated by the specific antigen to about the degree shown by selected cells in Table 1 (groups 3, 4, 6 or 7).

3.4 Cloning of antigen-specific T lymphoblasts

Cloning of antigen-selected T lymphoblast lines was done by limiting dilutions of cell numbers. The lymphoblast lines were diluted to 1 cell/well and cultured together with accessory cells and either BP or PPD. Table 3 shows an experiment in which we examined 384 microculture wells containing each antigen and found that about 30% of the cultures showed microscopic clumping of lymphoblasts, as evidence of a proliferative response, but only in the presence of the specific selecting antigen. There was no evidence of a proliferative response in the presence of the counterpart antigen to which the lymphocytes had not been selected.

Antigen specificity of the cloned lymphocytes was confirmed by measuring their proliferative response. One 96-well culture plate of each cloned well was measured by pulsing the wells with [^{3}H]dThd. Table 3 shows that the mean cpm of the wells that appeared positive on microscopical examination was significantly higher than that of the negative wells. Furthermore, there was no overlap in the range of cpm measured in the positive and the negative wells. Thus, the microscopical examination was a faithful representation of clonal proliferation. Furthermore, the cells in 20 clones that were strongly positive to either BP or PPD were expanded by propagation and restimulated by incubation of each antigen in the presence of accessory cells. Table 3 shows that these propagated clones demonstrated strict immunospecificity.

3.5 Anti-BP lymphoblast line mediates EAE

To test the functional characteristics of our T cell lines, we inoculated them i.v. into syngeneic Lewis rats. Table 4 shows that paralysis as a sign of EAE developed within 2–3 days in rats that received 10^6 or 25×10^6 cells of a line that was specifically reactive against BP. This line had been propagated *in vitro* for 4 months after selection against BP (Table 1, group 6). A lymphoblast line isolated from the same population of LN cells for reactivity to PPD (Table 1, group 7) was not active in mediating EAE (Table 4). Injection of 50×10^6 of the anti-PPD line failed to produce paralysis. A detailed analysis of the clinical course and histology of EAE mediated by T cell lines will be presented elsewhere.

4 Discussion

The procedure described here provides a relatively rapid method for selecting rat T lymphocytes with reactivity towards specific self or foreign antigens. The selected lymphocytes responded by incorporating [^{3}H]dThd in the presence of the specific antigen and MHC-compatible accessory cells (Table 2). The mitogen Con A also was able to activate antigen-selected lymphocytes. A population of LN lymphocytes that could initially respond to several different or cross-reactive antigens was dissected to select clones showing restricted responsiveness (Table 1). This suggests that the selection pro-

cedure favors the emergence of specific immunodominant clones and does not seem to carry along clones of lymphocytes that respond to unrelated, or even to cross-reactive, antigens. This finding is not easy to explain. We know that these other clones were present initially, and it seemed reasonable to assume that they would have been triggered to become lymphoblasts by nonspecific blastogenic factors [15, 16] elaborated by the antigen-selected lymphoblasts. Hence, we expected to find these other clones contaminating the specific populations isolated by density gradient fractionation. Nevertheless, such nonspecific lymphoblasts were not detected. Furthermore, expansion of single clones of selected lymphocytes also failed to reveal lymphocytes reactive against antigens present in the original immunization *in vivo* (Table 3). Preliminary experiments performed to analyze this paradox indicate that antigen-selected lymphoblasts might actively suppress other clones of lymphocytes in the population by means of extracellular factors (in preparation).

Although the mechanism of selection is not entirely clear, the selection procedure provides large quantities of clonable T lymphocytes that preserve their antigen specificity and can be used to approach a variety of questions. An important example is our finding that lymphoblasts selected to BP can mediate EAE (Table 4). I.v. injection of 10^6 selected lymphoblasts led to acute paralysis of Lewis rats within 2–3 days. This dose of selected T lymphocytes was at least two orders of magnitude less than the number of cells needed for passive transfer using unselected LN lymphocytes. Furthermore, the T cell line produced disease in less than one-half the time required using primed, unselected lymphocytes [17]. These findings indicate that T lymphocytes, apparently reactive against BP alone, are sufficient to mediate clinical EAE. The availability of this line now makes it feasible to study how such T lymphocytes actually produce paralysis. In addition, we have preliminary evidence that immunization of rats against the anti-BP T cell line can markedly decrease their susceptibility to induction of EAE. Hence, antigen-specific T cell lines might be used to alter the clinical course of autoimmune conditions.

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5 References

1 Binz, H. and Wigzell, H., *Cold Spring Harbor Symp. Quant. Biol.* 1976. *41:* 275.
2 Rajewsky, K. and Eichmann, K., *Contemp. Top. Immunobiol.* 1977. *7:* 69.
3 Zinkernagel, R. M., *Immunol. Rev.* 1978. *42:* 224.
4 Ruscetti, R. W., Morgan, D. A. and Gallo, R. C., *J. Immunol.* 1977. *119:* 131.
5 Gillis, S. and Smith, K. A., *Nature* 1977. *268:* 154.
6 Sredni, B. and Schwartz, R. H., *Nature* 1980. *283:* 581.
7 Von Boehmer, H., Hengartner, H., Nabholz, M., Lernhardt, W., Schreier, M. H. and Haas, W., *Eur. J. Immunol.* 1979. *9:* 592.
8 Paterson, P. Y., Drobish, D. G., Hanson, M. A. and Jacobs, A. F., *Int. Arch Allergy Appl. Immunol.* 1970. *37:* 26.
9 Wekerle, H., Ben-Nun, A., Hurtenbach, U. and Prester, M., in Rose, N. R., Bigazzi, P. E. and Warner, N. L. (Eds.), *Genetic Control of Autoimmune Disease,* Elevier/North-Holland Publishing Co., Amsterdam 1978, p. 415.
10 Rosenberg, S. A., Spiess, P. J. and Schwartz, S., *J. Immunol.* 1978. *121:* 1946.
11 Parish, C. R. and Hayward, J. A., *Proc. R. Soc. Lond. Ser. B* 1974. *187:* 379.
12 Schwartz, R. M., Yano, A. and Paul, W. E., *Immunol. Rev.* 1978. *40:* 153.
13 Pilarski, L. M., Bretscher, P. A. and Baum, L. L., *J. Exp. Med.* 1977. *145:* 1237.
14 Persson, U., Bick, P. H., Hammarström, L., Möller, E. and Smith, C. I. E., *Scand. J. Immunol.* 1978. *8:* 291.
15 Uotila, M., Rode, H. N. and Gordon, J., *Eur. J. Immunol.* 1978. *8:* 133.
16 Waldmann, H., Munro, A. and Hunter, P., *Eur. J. Immunol.* 1973. *3:* 167.
17 Paterson, P. Y., Richardson, W. P. and Drobish, D. G., *Cell. Immunol.* 1978. *16:* 48.