Brief Definitive Report

HAPTEN-SPECIFIC T CELL LINES MEDIATING DELAYED HYPERSENSITIVITY TO CONTACT-SENSITIZING AGENTS*

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T cells specific for a variety of antigens, including hapten-coupled cells (1, 2) and protein (3), have been cultured continuously in vitro. Although lines were shown to be hapten specific in that the presence of hapten was required, it has not, however, been possible to demonstrate that they could respond to hapten presented on different carriers. Such lines would be suitable for antigen- and anti-idiotype-binding studies as well as studies of fine specificity, such as heteroclicity. We cultured T cells from mice sensitized with the contact sensitizing agents 4-ethoxymethylene-2-phenyl oxazolone (OX) and picryl chloride (PCl) and demonstrated that, besides proliferating in response to antigen in vitro, the cells mediated delayed hypersensitivity (DH) to hapten presented on homologous or heterologous carrier.

Materials and Methods

Female CBA, BALB/c, BALB/c H-2^k, and (CBA × BALB/c)F_1 mice bred at the Water and Eliza Hall Institute were used between 6–12 wk of age.

Antigen-reactive and Antigen-presenting Cells. PCl (BDH Chemicals Ltd., Poole, England) and OX (BDH Chemicals Ltd.) were dissolved at 5 and 3% wt/vol, respectively, in ethanol, and a total volume of 100 μl was painted on the clipped thorax, abdomen, and forepaws of mice. For antigen-presenting cells, the inguinal, subscapular, and axillary lymph nodes were collected 24 h later and expressed through a stainless steel mesh in Hepes (10 mM, Sigma Chemical Company, St. Louis, MO) buffered Eagle's medium (Flow Laboratories, Inc.; Stanmore, Australia). The cell suspension was washed, irradiated (2,000 rad at 900 rad/min, with a Philips RT250 x-ray machine Philips Medical Systems Inc., Shelton, CT), and washed again. Antigen-reactive cells were collected from lymph nodes 3 d after painting, and cell suspensions were prepared similarly but not irradiated.

T Cell Culture Medium (TCM). Lymphocytes were cultured in RPMI (Flow Laboratories Inc.) containing 5×10^{-5} M 2-mercaptoethanol (Calbiochem-Behring Corp., American Hoescht Corp., San Diego, CA), 2 mM L-glutamine (Commonwealth Serum Laboratories, Melbourne, Australia), and 10% heat-treated fetal calf serum (Flow Laboratories, Inc.) supplemented with an ammonium sulphate-precipitated fraction from concanavalin A-stimulated spleen cell culture fluid (CAS) (4). CAS was prepared by culturing CBA spleen cells at 5×10^6 cells/ml in RPMI containing 1% fetal calf serum, 5×10^{-5} 2-mercaptoethanol, 1 mM L-glutamine, and 2 μg/ml concanavalin A (Calbiochem-Behring Corp. American Hoechst Corp.). After 24 h, the culture fluid was centrifuged at 400 *g* for 10 min and 800 *g* for 20 min to remove cells and debris, respectively. Concanavalin A was removed by precipitation with 40% ammonium sulphate and 0.1 M 2-α-methyl-*D*-mannoside (Sigma Chemical Company). The growth-stimulating fraction of CAS was then precipitated with 80% ammonium sulphate and the precipitate dialyzed against phosphate-buffered saline, pH 7.3 (4). Dilutions of this fraction were assayed and tested for their ability to support the survival of a CAS-requiring T cell line.

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Lymphocyte Cultures. 10^6 antigen-reactive cells and 10^6 irradiated antigen-presenting cells were cultured in 1.5 ml TCM per well in Costar trays (3524; Costar, Data Packaging, Cambridge, MA). After 3 d, cells were separated on a 1.077 density Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient (500 *g*, 20 min), and interface cells were washed and recultured in Costar trays with 10^5 antigen-reactive cells, 10^5 irradiated antigen-presenting cells, and 10^6 irradiated spleen cells in 1.5 ml per well TCM containing CAS. Each week, cells were separated on Ficoll gradients and restimulated as above. Cell yields decreased for 6–8 wk, then increased quickly to stabilize at a 10-fold increase per week.

Surface Marker Studies. Monoclonal rat anti-Thy-1.2 (30-H-12), anti-Ly-1 (53713), and anti-Ly-2 (53-6-7) (gifts from Dr. P. Bartlett of this Institute) were used for fluorescent staining using biotin-coupled antibody and fluorescein-coupled avidin (Sigma Chemical Co.) (5). Fluorescent cells were counted by fluorescent microscopy.

Proliferation Assays. Cells from cultures were taken 1 wk after stimulation with antigen without CAS and set up in Falcon microtiter wells at 5×10^4 cells per well with 5×10^4 irradiated antigen-presenting cells (see above) in TCM. After 48 h, 1 μCi [^{3}H]thymidine (Amersham Corp., Bucks, U. K.; 24 Ci/mmole) was added, and, after 16 h, radioactivity incorporated was determined by scintillation spectrometry.

Local DH Assay. The left ears of naive mice were painted with 10 μl of 0.5% oxazolone or PCl in a 50:50 vol/vol mixture of acetone dibutylphthalate. After 24 h, both ears were injected intracutaneously with 10 μl of cells (usually 5×10^5 cells per ear). Next day, ear thickness was measured with an engineer's micrometer, and the difference between left and right ears was used as a measure of DH and compared with ear thickness differences induced by normal lymph node cells.[1] Sensitivity to hapten-protein conjugates was tested by injecting 10 μl of 1 mg/ml of the conjugate 24 h before the cells. Trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) was prepared according to Rittenberg and Amkraut (6) (KLH, A grade, Calbiochem-Behring Corp.), and OX-KLH was prepared as described by Yoshimura and Cinader (7).

Results

Antigen-specific Proliferation. The three T cell lines described here have been maintained for up to 8 mo in culture. They include a BALB/c anti-OX line (05), a BALB/c anti-PCl line (P2), and a CBA anti-OX line (010). The three lines showed >97% of cells staining with anti-Thy-1.2, and the 05 and P2 line had 50 and 90% staining with anti-Ly-1 and no staining with anti-Ly-2. The 010 line did not stain above background with the anti-Ly reagents. All required both CAS and the appropriate antigenic stimulation for continued long-term culture. To test short-term proliferation, cells were cultured with antigen without CAS for 1 wk, then tested for proliferation using irradiated antigen-presenting cells from mice painted 24 h previously with OX or PCl. As shown for the 05 line (BALB/c anti-OX) (Table I), cells proliferated only when the appropriate antigen OX was presented by syngeneic or semisyngeneic cells. Proliferation could also be induced by mixtures of OX and PCl, showing that the failure of the 05 cells to proliferate in response to PCl was not because of any toxic effect of the PCl.

Contact Sensitivity. When tested for ability to induce a local DH, the cell lines 05 (BALB/c anti-OX), 010 (CBA anti-OX), and P2 (BALB/c anti-PCl) all produced large reactions, but only when challenged with the correct antigen (Table II). Although local DH reactions could be produced with cells taken from mice 3 d after painting, it usually required about 10–100 times the cell dose (Table II). The DH was MHC restricted, as shown by the ability of the CBA anti-OX line, 010, to produce

[1] These reactions showed DH kinetics because they persisted for at least 48 h. Measurements before 24 h were not made because of the trauma associated with cell inoculation directly into the ear.

TABLE I
Antigen Specificity and Genetic Restriction of Proliferation of a BALB/c Anti-OX T Cell Line (05)

Experiment	Antigen	Strain of antigen-presenting cells (5×10^4)	cpm ± SE*
(1)	OX	BALB/c	25,286 ± 1,290
	PCl	BALB/c	4,126 ± 324
	OX + PCl‡	BALB/c	31,528 ± 366
(2)	OX	BALB/c	106,002 ± 9,514
	OX	CBA	1,510 ± 300
	OX	CBA × BALB/c§	88,028 ± 8,360

* [^{3}H]Thymidine incorporation by 5×10^4 05 cells measured after 3 d.
‡ 5×10^4 PCl plus 5×10^4 OX antigen-presenting cells.
§ Cell line did not respond to uncoupled antigen-presenting F_1 cells.

TABLE II
Antigen-specific Contact Sensitivity

Experiment number	Cells injected into syngeneic recipients			Ear swelling (10^{-2} mm) in response to*	
	Type	Number	Challenge	Immune cells	Normal lymph node cells
1	BALB/c OX line 05	3×10^5	0.5% OX	16.2 ± 2.1	1.1 ± 1.3
		3×10^5	0.1% OX	8.1 ± 2.9	1.2 ± 0.6
		3×10^5	0.5% PCl	4.6 ± 1.1	3.1 ± 1.5
		3×10^5	0.1% PCl	1.4 ± 0.6	1.6 ± 0.5
2	BALB/c OX line 05	4×10^5	0.5% OX	8.0 ± 0.6	2.7 ± 0.4
		6×10^4	0.5% OX	7.3 ± 0.5	
		10^4	0.5% OX	4.9 ± 1.2	
3	OX-immune BALB/c	2.5×10^6	0.5% OX	6.2 ± 0.6	0.7 ± 0.7
	Lymph node cells‡	1.2×10^6	0.5% OX	5.4 ± 1.0	2.0 ± 0.8
4	CBA OX line 010	5×10^5	0.5% OX	18.1 ± 2.5	3.3 ± 1.3
		5×10^5	0.5% PCl	4.7 ± 1.3	7.4 ± 1.1
5	BALB/c PCl line P2	5×10^5	0.5% OX	2.4 ± 1.0	0.8 ± 0.8
		5×10^5	0.5% PCl	14.6 ± 2.0	4.9 ± 1.6

* Mean ± SE from groups of five mice.
‡ Draining lymph node cells 3 d after painting with 3% OX (70% Thy-1.2 positive).

DH in CBA, (CBA × BALB/c)F_1, BALB/c H-2^k, but not BALB/c mice (Table III), in contrast to the BALB/c 05 line, which produced DH in BALB/c but not in CBA mice.[2] This restriction allowed us to test the possibility that the irradiated antigen-presenting cells in culture might have contributed to the DH reactions. 010 (CBA anti-OX) cells were cultured for 1 wk with (CBA × BALB/c)F_1 antigen-presenting cells and filler cells and tested for DH production in CBA or BALB/c mice. DH reaction only occurred in CBA mice (Table IV).

Hapten-specific DH. Local DH assays were performed with OX-KLH or TNP-KLH. The anti-OX lines 05 and 010 responded to OX-KLH and the PCl line P2 to TNP-KLH (Table V).

[2] The difference in the responses of CBA and BALB/c H-2k noted in Table III has not been a consistent finding. Further experiments with an A.TL anti-OX line have shown the restriction primarily in the I/S region.

TABLE III

Genetic Restriction for Transfer of DH to OX

Cells injected (5×10^5/ear)	Recipient strain	Swelling (10^{-2} mm) in response to*	
		Cell line	Normal lymph node cells
010 (CBA)	CBA (H-2^k)	15.1 ± 3.1‡	1.4 ± 1.0
010 (CBA)	BALB/c (H-2^d)	2.7 ± 2.3	1.2 ± 0.6
010 (CBA)	BALB/c H-2^k (H-2^k)	7.5 ± 0.2‡	1.3 ± 0.6
010 (CBA)	(CBA × BALB/c)F_1	13.9 ± 0.9‡	1.6 ± 0.6
05 (BALB/c)	BALB/c	16.2 ± 1.6‡	2.6 ± 0.5
05 (BALB/c)	CBA	3.0 ± 0.8	3.0 ± 0.7

* Mean ± SE from groups of five mice.
‡ Significantly different from response of normal lymph node group; $P < 0.001$.

TABLE IV

Genetic Restriction of Transfer of DH by CBA 010 Anti-OX Line after Culture with (CBA × BALB/c)F_1 Antigen-presenting Cells

OX-challenged recipient	Ear swelling (10^{-2} mm) in response to*	
	010 line	Normal lymph node
CBA	23.4 ± 1.8‡	2.0 ± 0.5
BALB/c	0.4 ± 0.2	1.5 ± 0.3

* Mean ± SE from groups of five mice injected with 5×10^5 cells per ear.
‡ Significantly different from normal lymph node group; $P < 0.001$.

TABLE V

Hapten-specific DH

Cell line injected into syngeneic recipients	Challenge	Ear swelling (10^{-2} mm) in response to*	
		Cell line	Normal lymph node cells
010	OX-KLH	12.6 ± 1.3‡	2.4 ± 0.7
(CBA anti-OX)	TNP-KLH	3.8 ± 1.8	1.8 ± 0.4
05	OX-KLH	11.1 ± 2.2‡	1.6 ± 0.4
(BALB/c anti-OX)	TNP-KLH	2.4 ± 1.7	1.8 ± 2.0
P2	OX-KLH	4.2 ± 2.0	2.1 ± 1.0
(BALB/c anti-PCl)	TNP-KLH	8.1 ± 1.3‡	2.2 ± 0.6

* Mean ± SE from groups of five mice injected with 5×10^5 cells per ear.
‡ Significantly different from swelling produced by normal lymph node; $P < 0.01$.

Discussion

Cell lines specific for the contact-sensitizing agents OX and PCl have been established. For maintenance in vitro, the lines required both CAS and repeated antigenic stimulation (usually weekly). Antigen-specific proliferation was also shown in short-term assays by measuring [^{3}H]thymidine incorporation.

The three cell lines described all produced large swellings in local DH assays at cell doses about 100-fold less than required by uncultured immune cells. They showed appropriate antigen specificity for OX or PCl and were major histocompatibility complex (MHC) restricted, as shown by their activity in MHC-matched but not unmatched congenics or other mouse strains as well as by the activity of parental cells in F_1 mice with one unmatched MHC haplotype.

The antigen-presenting cells used to maintain the cultures were lymph node cells taken 1 d after painting mice with sensitizer. These cells have previously been shown (8, 9) to be exquisitely immunogenic when injected into mice and to contain T and B lymphocytes as well as macrophages and Langerhan's cells. Two observations have been made to exclude the possibility that the lymph node cells mediated the DH reactions. First, several clones were cultured with the antigen-presenting cells and did not produce DH. Second, after the CBA line 010 was cultured with (CBA × BALB/c)F_1 antigen-presenting cells, it could mediate DH only in CBA and not in BALB/c mice.

A feature of the lines is their hapten specificity, i.e., their ability to be activated by either hapten-self moieties or hapten conjugated to foreign protein. This was unexpected because studies (10) with guinea pigs showed that animals sensitized by contact sensitizers did not respond well to hapten presented on foreign carriers. Nevertheless, hapten-specific DH to 4-hydroxy-3-nitrophenyl acetyl (11) and azobenzene arsonate (12) have been reported in mice, and our own unpublished results with PCl and dinitrofluorobenzene have shown hapten specificity. Because of the experimental system used, cell lines prepared by others (2) against hapten-cell conjugates have not been able to differentiate between hapten-specific reactivity and reactivity to new antigenic determinants caused by haptenation of the cells. This has made it difficult to interpret experiments with T cell lines examining heteroclicity and idiotype expression. In contrast, in vivo experiments (13) with T cells from mice expressing hapten-specific DH elicited by 4-hydroxy-3-nitrophenyl acetyl on different carriers have shown heteroclicity and V_H-linked control of specificity. In this regard, it has been shown (14) that BALB/c anti-OX antibodies are relatively homogeneous and have a defined idiotype.

The ability of the lines to mediate other T cell functions has not yet been examined. Because some but not all cytotoxic T cell clones can mediate DH (15, 16), as can T helper cell clones (17), it would be pertinent to test for other functions to determine whether or not unique DH T cells exist. It is perhaps of interest that all clones grown from these lines to date were inactive, indicating a possible requirement for cellular interaction to produce lines capable of mediating DH.

Summary

Continuous cultures of T cells from the lymph nodes of mice sensitized to the contact sensitizers 4-ethoxymethylene-2-phenyl oxazolone or picryl chloride have been established. For continuous proliferation, the lines required specific antigen, syngeneic antigen-presenting cells, and growth factors from the supernatant of concanavalin-A-stimulated lymphoid cultures. Cells from the lines showed antigen specificity and major histocompatibility complex restriction in proliferation assays and in delayed hypersensitivity. They could mediate delayed hypersensitivity to the sensitizer presented as a reactive hapten or coupled to keyhold limpet hemocyanin.

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