Brief Definitive Report

SPECIFIC Lyt 123 T CELLS ARE INVOLVED IN PROTECTION AGAINST *LISTERIA MONOCYTOGENES* AND IN DELAYED-TYPE HYPERSENSITIVITY TO LISTERIAL ANTIGENS*

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The use of Lyt antisera against cell-surface differentiation antigens in mice led to the generalization that the functional diversity of T cells correlates with characteristic cell-surface patterns of Lyt antigens. Thus, helper T cells and effector T cells mediating delayed-type hypersensitivity (DTH) have been reported to be of the Lyt $1^+2^-3^-$ phenotype, whereas cytotoxic and suppressor T cells were shown to belong to the Lyt $1^-2^+3^+$ subclass (1). The function of the third subset of T cells, Lyt 123^+, is less well understood, but it is assumed that they serve as precursors of the Lyt 1^+ and Lyt 23^+ subclasses (1). Recent studies have revealed that this population also consists of regulatory cells (2) as well as precursors of H-2-restricted and alloreactive effector cells (3).[1]

Although it has been known for a long time that T cells play a central role in immunity to facultatively intracellular bacteria (4, 5), the Lyt phenotype of the relevant subpopulation(s) has not yet been established. We have therefore examined the Lyt phenotype of committed peritoneal exudate T lymphocytes (PETLs) functional in protection against *Listeria monocytogenes* and in DTH to listerial antigens.

Materials and Methods

Mice. Female C57BL/6 mice were obtained from Iffa Credo, l'Arbresle, France, or from Zentralinstitut für Versuchstierzucht, Hannover, Federal Republic of Germany, and used at 8 wk of age.

Bacteria and Bacterial Antigens. *L. monocytogenes* (strain EGD) was kept virulent by continuous mouse passage. Cultures were obtained by growing a sample of spleen homogenate from an infected mouse in trypticase-soy broth (Oxoid, Wesel, Federal Republic of Germany). Bacterial suspensions were appropriately diluted in 0.15 M NaCl and injected i.v. in a vol of 0.2 ml. The number of *L. monocytogenes* injected was confirmed by plate count according to (6).

For soluble antigens of *L. monocytogenes*, bacteria were cultured for 3 d in an ultrafiltrate ($\leq$ 10,000 mol wt, Diaflo Hollow Fiber H1P10, Amicon Corp. Scientific Sys. Div., Lexington, Mass.) of trypticase-soy broth. The culture medium was centrifuged at 10,000 *g* and the supernate concentrated 200-fold ($\geq$ 10,000 mol wt, Diaflo Hollow Fiber H1P10). The concentrate was dialyzed thoroughly against distilled water, lyophilized, and stored at −70°C. The lyophilisate was dissolved in 0.15 M NaCl (5 mg/ml) and sterile-filtered immediately before use.

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[1] Simon, M. M., and B. Abenhardt. Generation of effector cells from T cell subsets. II. Lyt 123 T cells are the source of precursors for alloreactive and H-2 restricted killer cells and contain cells involved in the regulation of cytotoxic responses. Submitted for publication.

Immunizations and Enrichment for PETLs. Prospective donor mice were immunized with 5×10^3 live *L. monocytogenes*. 6 d later, peritoneal exudates were induced with 2 ml of 5% casein and cells harvested 48 h later as previously described (7). Peritoneal exudate cells were enriched for PETLs by separation on nylon-wool columns (8), washed, counted, and used either as unselected population or treated with antisera plus complement (see below).

Adoptive Transfer of Antibacterial Protection and DTH. Recipient mice were injected i.v. with 3×10^6 unselected PETLs or with equivalent numbers of selected Lyt 1^+, Lyt 23^+, or Lyt 1^+ plus Lyt 23^+ lymphocytes. At the same time, the animals were infected with 5×10^4 *L. monocytogenes* 48 or 72 h later, bacteria were counted in spleens by plating 0.1 ml samples of spleen homogenate at appropriate dilutions on trypticase-soy agar as described (6). For adoptive transfer of DTH, recipient mice were injected i.v. with 3×10^6 unselected T cells or T cells selected for Lyt subpopulations and challenged by s.c. injection of 50 μl antigen solution into one hind footpad. Footpad thicknesses were measured 24 h later using a dial gauge caliper (Kröplin, Schlüchtern, Federal Republic of Germany) according to (9).

Antisera and Treatment of PETLs with Antisera. Anti-Thy 1.2 antiserum (AKR anti-C3H) was kindly provided by Dr. B. Rubin, Statens Seruminstitut, Copenhagen, Denmark. Anti-Lyt antisera were prepared and absorbed to remove autoantibodies as described by Shen et al. (10). 3×10^7 PETLs/ml were treated at room temperature for 30 min with anti-Thy 1.2 antiserum (final dilution 1:20), anti-Lyt 1.2 antiserum (final dilution 1:20), or anti-Lyt 2.2 antiserum (final dilution 1:20) plus anti-Lyt 3.2 antiserum (final dilution 1:40), respectively. Cells were washed, resuspended at a concentration of 3×10^7/ml in selected rabbit serum as a source of complement (final dilution 1:11), and kept for 30 min at 37°C. Treatment of cells with anti-Lyt antisera and complement was repeated once. After treatment, cell samples were adjusted to volumes equivalent to those of control cultures treated with complement alone. Populations consisting of Lyt 1 and Lyt 23 cells were a 1:1 mixture of lymphocytes treated either with anti-Lyt 1.2 or anti-Lyt 2.2 and anti-Lyt 3.2 antisera plus complement. Cell numbers were not adjusted for differential viability resulting from antiserum treatment. However, appropriate dose-response experiments were done to ensure that cell numbers as left after antiserum treatments would have been sufficient for transferring substantial degrees of protection and DTH (data not shown).

Mixed Lymphocyte Cultures and Assay for Cytotoxic Activity Generated In Vitro. Equivalents of 0.6×10^6 unselected C57BL/6 PETLs or PETLs selected for Lyt T-cell subsets were cocultured for 4 d with 3×10^6 x-irradiated stimulator cells in 2 ml RPMI-medium (Gibco Bio-Cult Ltd., Paisley, United Kingdom) supplemented with L-glutamine (2 mM), streptomycin (50 μg/ml) and penicillin (50 U/ml), Hepes buffer (25 mM), 2-mercaptoethanol (2×10^{-5} M), and 10% fetal calf serum in Linbro macrotiter plates (Linbro Chemical Co., Hamden, Conn.; catalog No. FB 16-24 TC). On day 2, triplicates of 100 μl from each culture were labeled with [^{3}H]-thymidine (2 Ci/ml, Radiochemical Centre, Amersham-Buchler, Amersham, England), at 2 μCi/well for 12 h. Specific incorporation was determined by subtracting mean background responses of responder cells incubated with irradiated syngeneic cells.

Cell-mediated cytotoxicity generated in the same cultures was assayed on day 4. Effector cells from individual cultures were tested in a 4-h assay on ^{51}Cr-labeled P815 tumor cells (H-2^d) at the effector to target-cell ratios indicated. The percentage of ^{51}Cr released from target cells was determined using triplicate samples and calculated using the following formula: percentage of specific lysis = $100 \times (a - b)/(c - b)$ (a, ^{51}Cr release by immune cells, b, ^{51}Cr release by target cells alone, c, maximal ^{51}Cr release from target cells).

In Vitro Generation of Sheep Erythrocyte-specific Plaque-forming Cells. Equivalents of 0.6×10^6 unselected C57BL/6 PETLs or PETLs selected for Lyt T-cell subsets were incubated for 4 d in Falcon tissue culture plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) containing 0.3 ml Click's medium and 3×10^6 purified C57BL/6 B cells obtained by treating spleen cells with anti-Thy 1.2 antiserum and complement. The cultures were sensitized with sheep erythrocytes (SRBC), incubated at 37°C in humidified air plus CO_2, and assayed 4 d later for anti-SRBC plaque-forming cells (PFC) according to (11).

Results

Specificity of Anti-Lyt Antisera. To ascertain that the anti-Lyt antisera used in the experiments indeed selected for the appropriate T-cell subsets, PETLs from the same

TABLE I

Effect of Anti-Lyt Antisera and Complement on the Capacity of PETLs from L. monocytogenes-immune Mice to Generate Helper Functions in a Primary Antibody Response to SRBC and on the Proliferative Responses to Alloantigens in a Mixed Lymphocyte Reaction In Vitro

Group	Treatment	T-cell subsets	Cooperation of PETLs and splenic B cells in a primary anti-SRBC PFC response in vitro*			Proliferative response of PETLs to H-2^d alloantigens‡
			B cells (3 × 10^6)	SRBC	Mean PFC/10^6 B cells	
						mean cpm
1	—	Unselected	+	+	96	66,590
2	Complement	Unselected	+	+	167	88,427
3	Anti-Lyt 1.2 + complement	Lyt 23$^+$	+	+	39	7,830
4	Anti-Lyt 2.2, anti-Lyt 3.2 + complement	Lyt 1$^+$	+	+	111	41,103
5	Mixture of treatments of groups 3 and 4 (1:1)	Lyt 1$^+$ + Lyt 23$^+$	+	+	52	48,595
6	—	No	+	−	0	—
7	—	No	+	+	0	—

* Equivalents of 0.6 × 10^6 PETLs from the same *L. monocytogenes*-immune mice as in experiment II in Table II.
‡ 3 × 10^6 x-irradiated DBA spleen cells were used as stimulator cells.

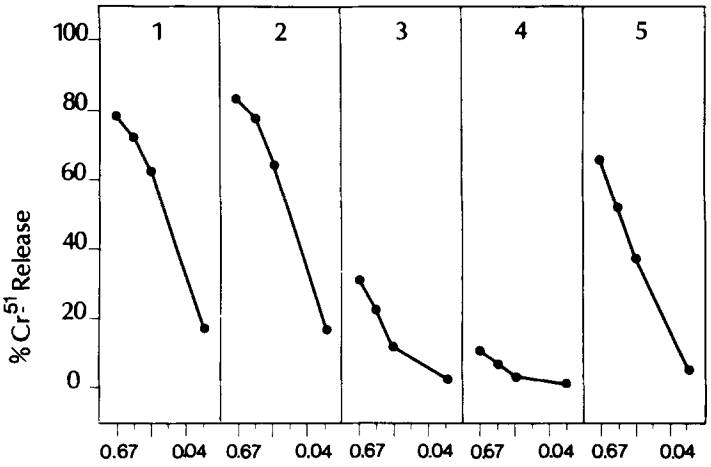

FIG. 1. Effect of anti-Lyt antisera and complement on the capacity of PETLs from *L. monocytogenes*-immune mice to generate cytotoxic responses to alloantigens in vitro. The same cells as in the experiment in Table I were used. Abscissa: number of responder cells (× 10^6) cultivated on day 0 (see Table I) the descendants of which were tested for killer activity on day 4 in a 4-h assay on 1 × 10^4 ^{51}Cr-labeled P815 target cells. 1: untreated PETLs; 2: complement-treated PETLs; 3: PETLs treated with anti-Lyt 1.2 antiserum + complement; 4: PETLs treated with anti-Lyt 2.2, anti-Lyt 3.2 antiserum + complement; 5: mixture of treatments of groups 3 and 4 (1:1).

cell pool obtained from *L. monocytogenes*-immune mice as that used for adoptive transfer of protection and DTH were pretreated with anti-Lyt antisera and complement and tested for their ability to cooperate with B cells in a primary antibody response to SRBC in vitro. In addition, the selected Lyt subpopulations were tested for their proliferative responses to alloantigens in a mixed lymphocyte reaction and for their ability to generate alloreactive cytotoxic activity in vitro. As shown in Table I, the primary anti-SRBC response in vitro was markedly reduced after pretreatment of PETLs with anti-Lyt 1.2 antiserum, but not with anti-Lyt 2.2 and anti-Lyt 3.2

TABLE II

Effect of Anti-Lyt Antisera and Complement on the Capacity of PETLs from L. monocytogenes-immune Mice to Transfer Protection against L. monocytogenes and DTH to Listerial Antigens

Group	Treatment of PETLs	T-cell subsets	Experiment I		Experiment II	
			$\log_{10}$ viable bacteria in spleens (48 h)*	DTH (0.1 mm)*	$\log_{10}$ viable bacteria in spleens (72 h)*	DTH (0.1 mm)*
1	None	Unselected	3.48 (± 0.85)	8.8 (± 2.3)	4.06 (± 0.52)	9.0 (± 2.7)
2	Complement	Unselected	4.57 (± 0.31)	9.2 (± 1.3)	4.27 (± 0.89)	8.0 (± 1.3)
3	Anti-Thy 1.2 + complement	No	6.51 (± 0.54)	2.4 (± 0.55)	ND	ND
4	Anti-Lyt 1.2 + complement	Lyt 23^+	6.54 (± 0.41)	2.2 (± 0.45)	5.78 (± 0.52)	2.8 (± 0.84)
5	Anti-Lyt 2.2, anti-Lyt 3.2 + complement	Lyt 1^+	6.38 (± 0.71)	4.4 (± 1.5)	5.38 (± 0.36)	4.3 (± 0.82)
6	Mixture of treatments of groups 4 and 5 (1:1)	Lyt 1^+ + Lyt 23^+	6.52 (± 0.64)	4.0 (± 0.71)	5.68 (± 1.29)	3.8 (± 1.7)
7	No PETLs	—	7.16 (± 0.53)	2.2 (± 0.45)	5.63 (± 0.27)	1.7 (± 0.82)

* Equivalents of 3×10^6 PETLs from *L. monocytogenes*-immune donor mice; means of six animals per group ± SEM. Significant differences ($P \leq 0.05$) against groups 1 and 2: groups 3, 4, 5, 6, and 7.

antisera and complement. Furthermore, the proliferative response to alloantigens of selected Lyt 23^+, but not that of selected Lyt 1^+, PETLs was drastically reduced (Table I). The generation of alloreactive cytotoxic lymphocytes from PETLs was abolished in populations pretreated with anti-Lyt 2.2 and anti-Lyt 3.2 antisera or anti-Lyt 1.2 antiserum plus complement, but could be restored by admixing Lyt 1^+ and Lyt 23^+ PETLs (Fig. 1). Thus, in accordance with published evidence (1), the anti-Lyt antisera were of the predicted specificities. In addition, the experiments revealed that PETLs from *L. monocytogenes*-immune mice encompass a mixture of distinct T-cell subpopulations including precursor cells able to develop into effector cells with diverse immunological activities.

Lyt Phenotype of Protective and DTH-transferring PETLs. The data depicted in Table II show that protective activity against *L. monocytogenes* and DTH reactions to listerial antigens could not be transferred by specific PETLs treated with either anti-Lyt 1.2 (Lyt 23^+ subset) or anti-Lyt 2.2 and anti-Lyt 3.2 (Lyt 1^+ subset) antisera and complement, respectively, nor by the mixed population consisting of Lyt 1^+ and Lyt 23^+ cells. Both activities were detected only after transfer of unselected immune PETLs consisting of Lyt 123^+ cells in addition.

Discussion

Our findings show that the specific T lymphocytes involved in protection against *L. monocytogenes*, a facultatively intracellular bacterium, and in DTH reactions to listerial antigens are of the Lyt 123^+ phenotype. Because effector T cells mediating DTH reactions to SRBC and fowl gamma globulin have been reported to be phenotypically Lyt 1^+23^- (9, 12, 13), our data imply either that particular T-cell functions are not restricted to particular Lyt T-cell subpopulations or that different kinds of antigen induce different pathways of T-lymphocyte activation, or both (14, 15). The participation of Lyt 123^+ T cells in DTH generated during the course of an infection can be taken as further evidence for the existence of two pathogenetically distinct types of DTH postulated for a long time (16): in one type of DTH, which is evanescent and is induced by noninfectious agents such as SRBC (Jones-Mote type),

Lyt 1^+23^- T cells participate, whereas in the other type, which is induced by intracellular bacteria (tuberculin-type), mainly Lyt 123^+ T cells are involved.

Whether, in addition to Lyt 123^+ cells, Lyt 1^+ T cells participate in DTH reactions to listerial antigens was not ruled out in these experiments. Indeed, although statistically not significant, treatment of PETLs with anti-Lyt 2.2 and anti-Lyt 3.2 antiserum did not as completely abrogate DTH reactions as did treatment of PETLs with anti-Lyt 1.2 antiserum (Table II, group 5 vs. group 4). However, this point needs further clarification.

The involvement of Lyt 123^+ T cells in cellular protection to *L. monocytogenes* and in DTH to listerial antigens could take place in at least three alternative ways. First, both phenomena are mediated by effector T cells of the Lyt 123^+ subset alone. Second, protective activity and DTH reactions are mediated by different T-cell subsets (Lyt 1^+ and/or Lyt 23^+), but require the assistance of Lyt 123^+ cells. Indeed, participation of Lyt 123^+ cells in positive and negative regulation of the immune response has been reported in several systems (2, 17).[1] Third, Lyt 123^+ precursors after transfer to recipients mature to effector cells carrying the Lyt 1^+ or the Lyt 23^+ phenotype (1, 3). Such a differentiation pathway has been shown to occur in vitro during the generation of H-2-restricted trinitrophenyl-specific and alloreactive killer cells.[1] Although our data do not distinguish between these alternatives, they clearly demonstrate the involvement of Lyt 123^+ cells in the in vivo cellular immune response against infections with the intracellular bacterium *L. monocytogenes*, at least if the former are derived from peritoneal exudates.

Summary

Specific anti-Lyt antisera and complement were used to determine the Lyt phenotype of peritoneal exudate T lymphocytes from *Listeria monocytogenes*-immune mice. It was found that Lyt 123^+ T cells are crucially involved both in protection against listerial infection and in delayed-type hypersensitivity (DTH) to listerial antigens. Thus, both functions critically depend on a T-cell subclass phenotypically different from that which mediates DTH to noninfectious antigens and help in antibody formation on the one hand, as well as those T cells mediating cytotoxic reactions on the other.

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