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Francisella tularensis-Specific T-Cell Clones Are Human Leukocyte Antigen Class II Restricted, Secrete Interleukin-2 and Gamma Interferon, and Induce Immunoglobulin Production

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T-cell clones (TLC) were established from a *Francisella tularensis*-vaccinated subject in order to study the cells responsive for cell-mediated immunity against *F. tularensis*. All the clones were human leukocyte antigen (HLA) class II restricted to one of the HLA-DR specificities of the original donor. The TLC cells were $CD4^+$ and produced interleukin-2 and gamma interferon after stimulation with specific antigen. Seven of the eight clones tested assisted in the production of immunoglobulin G (IgG), IgA, and IgM antibodies.

Cell-mediated immunity is important for protection against infections caused by bacteria which survive and grow intracellularly. T lymphocytes are specific effectors of cell-mediated immunity in that they recognize microbe antigens and produce mediators such as interleukin-2 (IL-2) and gamma interferon (IFN-γ) for the recruitment of mononuclear phagocytes (6). T lymphocytes with the $L3T4^+$ (CD4) phenotype are responsible for long-term cell-mediated immunity to *Mycobacterium tuberculosis* in the mouse (19), but there is recent evidence that suppressor-cytotoxic cells are also important for mycobacterial immunity (5, 17).

Immunity to the infection caused by *Francisella tularensis*, tularemia, is mainly cell mediated both in humans (24) and in animals (1, 13). Effective protective immunity can be induced by a live vaccine strain of *F. tularensis* (7) and can be transferred to nonimmune animals in T lymphocytes (1, 13). T lymphocytes respond to the whole bacterial antigens in vitro (25) and also to protein antigens (22; Surcel et al., manuscript in preparation), which are thought to be important for inducing cell-mediated immunity. Both IL-2 and IFN-γ are produced in the lymphocyte cultures stimulated by *F. tularensis* antigen, along with DNA and IL-2 receptors (10).

We characterize here the nature and function of tularemia-immune T lymphocytes by culturing T-cell clones (TLC) from a subject with human leukocyte antigen (HLA) class II antigens DR1,2;DQw1 who had been immunized with a live vaccine 3 years earlier.

F. tularensis LVS (BB IND 157.6111) bacteria used in this study were supplied by the U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, Md., and were cultured on medium consisting of 1.5% (wt/vol) Proteose Peptone no. 3 (Difco Laboratories, Detroit, Mich.), 55.5 mM glucose, 85.5 mM NaCl, and 0.2 mM $FeSO_4 \cdot 7H_2O$ in 54 mM K_2HPO_4–13 mM KH_2PO_4 buffer (pH 7.4) supplemented with IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). The bacteria were harvested at the early logarithmic growth phase by centrifugation at 10,000 × *g* for 5 min at 4°C and killed by treatment with 0.1% formaldehyde for 4 h at 25°C. They were then washed 8 to 10 times with physiological phosphate buffer (PBS Dulbecco; GIBCO, Scotland) and suspended in this buffer at the optimal protein concentration for use as an antigen in the lymphocyte blast transformation test.

T-lymphocyte clones were obtained from peripheral blood mononuclear cells of an HLA-DR1,2;DQw1-positive *F. tularensis*-vaccinated volunteer. HLA-DR antigens were determined by a standard two-stage microlymphocytotoxicity test with commercial HLA-DR trays (Biotest Serum Institute, Frankfurt/MW, Federal Republic of Germany). Peripheral blood mononuclear cells were separated by Lymphoprep (Nyegaard & Co. a/s, Oslo, Norway) gradient centrifugation and suspended (0.25×10^6/ml) in RPMI 1640 medium (GIBCO) supplemented with 10% pooled human AB serum and stimulated with *F. tularensis* antigen (1.0 μg/ml) for 7 days in a humidified 5% CO_2 atmosphere at 37°C. The blast cells were enriched on a gradient of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) after centrifugation (1,000 × *g*, 10 min) at the interface between 50 and 60% densities, washed twice with Hanks balanced salt buffer, suspended in RPMI 1640 medium supplemented with 10% AB serum and 10% IL-2 (Biotest Serum Institute), and cloned on Terasaki plates by using the limiting-dilution method. Briefly, 0.5 blast cells per well were cultured for 10 days with *F. tularensis* antigen and with fresh irradiated autologous peripheral blood mononuclear cells (10,000 cells per well) as the antigen-presenting cells (APC). Thereafter, the proliferating TLC (the cloning efficacy was 15 to 20%) were collected and transferred to microtiter plates and restimulated with 10^5 fresh APC and antigen as above. IL-2 was added to the growing clones at 2- to 3-day intervals, and the clones were restimulated at 10-day intervals.

The antigen specificity of the proliferating clones was tested after 4 to 5 weeks of blast enrichment by culturing 10^4 TLC in triplicate wells for 48 h on 96-well round-bottom microtiter plates in the presence of 10^4 APC and antigen suspended in RPMI 1640 supplemented with 10% AB serum with a total volume of 200 μl. [^{3}H]thymidine was present in the cultures for the last 18 h of the incubation, and proliferation was assayed from the incorporated radioactivity measured by using a liquid scintillation counter (LKB Wallac, Turku, Finland). The results are expressed in terms of median numbers of counts per minute. A clone was considered nonspecific when its proliferation response to tetanus toxoid had a stimulation index in excess of one-half of the response to *F. tularensis* antigen. The nonspecific clones were discarded.

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TABLE 1. Responses of *F. tularensis*-specific TLC in the presence of fresh APC sharing various class II HLA specificities with the T-cell donor

Clone no.	Tularemia response (cpm) to:					
	Autologous APC[a]	DR1	DR1	DR2	DR2	DQw1
1	2,508	5,372	4,267	406	358	431
2	10,358	10,074	7,559	74	179	99
3	4,298	7,947	5,993	173	257	178
4	5,206	114	185	9,865	8,889	290
5	9,593	162	760	16,193	19,085	870
6	1,935	498	359	3,563	2,381	698
7	4,208	6,144	6,402	1,411	200	959
8	2,008	176	485	4,931	4,460	354
9	3,590	5,451	4,910	342	122	306
10	3,262	129	232	7,822	7,767	231
11	6,364	90	565	12,760	13,494	654
12	3,146	147	91	7,231	3,890	520
13	2,746	5,771	4,872	380	321	221
14	4,278	81	377	7,159	8,879	246
15	2,290	59	224	6,360	6,194	230
16	2,802	142	562	8,182	7,263	386
17	1,754	122	248	3,893	3,832	171
18	1,270	79	89	1,892	2,264	78

[a] TLC responses to tetanus toxoid in the presence of autologous APC were <100 cpm.

Surface antigens of the clones were studied by direct immunofluorescence by using fluorescein-conjugated monoclonal anti-CD4 (T helper) and CD8 (T suppressor-cytotoxic) antibodies (Anti-Leu3 and Anti-Leu2; Becton Dickinson, Mountain View, Calif.).

The IL-2 and IFN-γ supernatants were obtained from 10^5 TLC suspended in a total of 1 ml of RPMI 1640 medium in culture tubes with 2×10^5 irradiated autologous APC and antigen and incubated in a humidified 5% CO_2 atmosphere at 37°C. The supernatants were collected after 24 h (for IL-2) or 48 h (for IFN-γ) and kept frozen until analysis.

IL-2 was measured by an IL-2-dependent mouse cytotoxic T-cell line (CTLL) as the responder cells in a proliferation assay. A total of 5,000 CTLL cells were cultured for 48 h with RPMI 1640 medium diluted 1:2 or 1:4 with the supernatant to be tested. The cells were pulsed with [^{3}H]thymidine for the last 18 h of incubation and harvested as described before. IFN-γ was analyzed by using an enzyme-linked immunosorbent assay as described previously by Karttunen et al. (10).

To study the TLC-induced antibody secretion, 2×10^5 TLC and 2×10^5 autologous non-T cells in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO) were stimulated in triplicate 200-μl wells on 96-well microtiter plates. The number of cells secreting antibodies (immunoglobulin M [IgM], IgG, IgA) was determined by enzyme-linked immunosorbent spot assay as described by Ryhänen et al. (20).

In the first experiment, 18 *F. tularensis*-specific clones were tested for HLA restriction. Clones restricted for DR1 or DR2 specificities were obtained, but *F. tularensis* antigen was not recognized in the context of DQw1 (Table 1). In a new series of similar TLC from the same subject, eight TLC were cultured for further analysis. The *F. tularensis*-specific clones all had the T helper phenotype ($CD4^+$, $DC8^-$). On stimulation with specific antigen, all the eight clones tested secreted IL-2 and IFN-γ (Table 2) and repeatedly induced the production of at least two immunoglobulin classes (Fig. 1).

TABLE 2. IL-2 and IFN-γ production of *F. tularensis*-specific TLC stimulated by original antigen presented by the autologous APC

Clone no.	Tularemia response (cpm)	IL-2 production (cpm)[a]	IFN-γ production (IU/ml)[b]
19	7,202	5,004	535
20	12,120	4,541	198
21	4,874	1,158	446
22	7,022	2,941	286
23	6,191	3,222	275
24	6,469	2,759	740

[a] IL-2 production induced by tetanus toxoid was <100 cpm.
[b] IFN-γ production induced by tetanus toxoid was <33 IU/ml.

The aim of this work was to examine at the single-cell level the functional properties of peripheral blood T lymphocytes responding specifically to *F. tularensis* bacterial antigen. A limiting-dilution microculture system was used to culture TLC from a healthy *F. tularensis*-vaccinated subject. The true clonal nature of the obtained lines was not proven, and the possibility remains that some of them represent lines derived from multiple responding cells. However, the clonality can be assumed in most cases because of the original limiting dilution with a cloning efficacy of 15 to 20%. Also, the selected clones were all restricted by either DR1 or DR2 specificity.

The antigen response of a T lymphocyte is initiated by the interaction of the specific T-cell receptor with both the nominal antigen and the autologous major histocompatibility complex molecule at the surface of the antigen-presenting cell. $CD4^+$ T cells are restricted to major histocompatibility complex class II antigens, and $CD8^+$ T cells are restricted to major histocompatibility complex class I antigens. Both major T-cell subclasses may play a role in protection against intracellular bacterial infections. $CD8^+$ cytotoxic T cells have been shown to be protective against *Mycobacterium lepraemurium* (8) and *Plasmodium falciparum* in mice (14). Both $CD4^+$ and $CD8^+$ T cells seem to be relevant to antibacterial immunity in murine listeriosis (11), although $CD8^+$ T cells have been demonstrated to be the major effectors of bacterial clearance, especially in a secondary immune response (16).

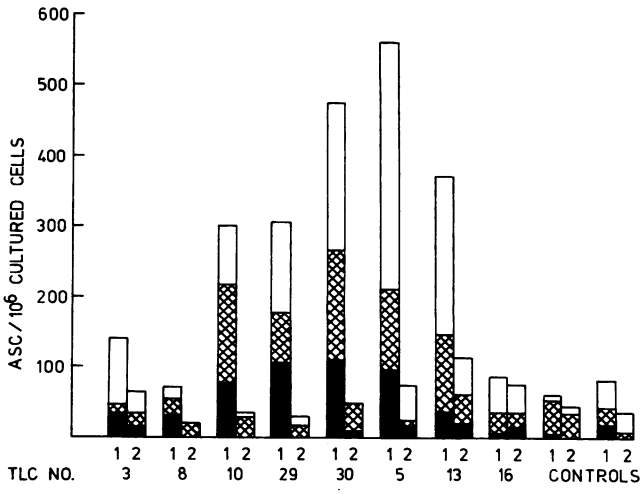

FIG. 1. Number of antibody IgM (■)-, IgG (▩)-, or IgA (□)-secreting non-T-cells induced by *F. tularensis*-specific TLC after stimulation with *F. tularensis* antigen (1) or tetanus toxoid (2).

The occurrence of $CD8^+$ cells in peripheral blood may be limited to the acute phase of the disease, as shown in patients recently infected with *P. falciparum* (23). Antigen-specific T-cell clones from healthy malaria-immune subjects were all of the $CD4^+$ subgroup, supporting the notion that it is the $CD4^+$ T cells that are responsible for specific long-term immunity. $CD4^+$ helper T cells may in turn also promote the formation of $CD8^+$ cell-mediated effector mechanisms.

The *F. tularensis*-specific human TLC raised here from a healthy *F. tularensis*-immune subject were all $CD4^+$ restricted by HLA-DR. The result is comparable with tularemia immunity in mice, for which the antigen-specific T-cell response has recently been described as requiring major histocompatibility complex class II-restricted presentation (2).

In mouse lymphocytes, a dichotomy among $CD4^+$ T cells according to specificity and function into helper T and inflammatory T cells is suggested. Inflammatory T cells are postulated to mediate surveillance of intracellular pathogens, and helper T cells are postulated to be specialized in self-non-self discrimination among extracellular antigens (4). The *F. tularensis*-specific $CD4^+$ TLC raised here release IL-2 and IFN-γ, resembling the T cells found to mediate immunity against *M. tuberculosis* in mice (3, 12) and against *M. bovis* in humans (18).

In addition to specific antibody production, microbes induce polyclonal immunoglobulin production (15), in which T-cell help is provided by specific inflammatory T cells (9). We used the enzyme-linked immunosorbent spot assay method to analyze the capacity of specifically stimulated TLC to induce B cells to produce antibodies. IgG-, IgA-, and IgM-class immunoglobulins were produced in the presence of the specific antigen and a TLC sharing the known HLA restriction specificity with B-cell donors. The specificity of the antibody produced was not tested, however.

Quite even amounts of both DR1- and DR2-restricted clones were obtained from the HLA-DR1,2;DQw1-positive donor. This could be expected, as the clones were raised against a complex bacterial structure that includes numerous antigenic epitopes (21). The use of defined antigenic fractions to test T-cell clone responsiveness and HLA restriction may reveal possible specific associations between antigenic structure and HLA class II specificities which can be used to elicit an immune response.

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