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Several Membrane Polypeptides of the Live Vaccine Strain *Francisella tularensis* LVS Stimulate T Cells from Naturally Infected Individuals

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The currently used live vaccine strain *Francisella tularensis* LVS was derived several decades ago from a wild strain of the species. In the present report, several membrane polypeptides of LVS are shown to be recognized by T cells from individuals immunized by natural infection with *F. tularensis*. Bacterial membranes of a capsule-deficient mutant of LVS were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Thereafter, gels were divided into seven fractions, each fraction containing a different number of peptide bands. From other gels, four bands were excised, each containing one major polypeptide. Eluates of each fraction and of each polypeptide band induced a proliferative response and an interleukin-2 response in lymphocytes from most of the individuals. When the lymphocytes were separated after induction, most of the proliferative response was found to occur in $CD4^+$ T cells. Lymphocytes from nonimmune individuals responded poorly to all membrane polypeptides. To study the possible heterogeneity of antigen determinants among the polypeptides, T-cell clones were raised towards *F. tularensis* and tested for proliferative response to the four major membrane polypeptides. Five clones, all $CD4^+$ $CD8^-$, responded to one or more of the polypeptides, each clone with a unique pattern of response. In conclusion, *F. tularensis* possesses a high number of T-cell-reactive membrane polypeptides. There seems to be a heterogeneity of T-cell determinants among these polypeptides. Determinants involved in immunization by natural infection are well conserved in LVS.

Francisella tularensis is a facultative intracellular bacterium. It is highly virulent, and only a few organisms are required to cause tularemia in humans (22, 23). Host protection against tularemia is induced by natural infection or by vaccination with viable attenuated bacteria but not by vaccination with killed bacteria (3, 8, 22, 23, 26). This is similar to the induction of host protection against other facultative intracellular parasites.

In 1956, a mixture of strains of *F. tularensis* with attenuated virulence was transferred from the Soviet Union to the United States (26). From this mixture, one strain was selected, tested for safety and efficacy in intradermal vaccination, and introduced as the live vaccine strain *F. tularensis* LVS (4). Vaccination with LVS affords good protection against the respiratory form of tularemia and modifies signs and symptoms of the ulceroglandular form of the disease (3).

Cell-mediated immunity is a prerequisite of the host defense against *F. tularensis*, whereas humoral immunity is of minor importance (10). The ability to induce cell-mediated immunity resides in membrane proteins of the bacteria. Four major membrane polypeptides of a capsule-deficient strain of LVS all induced a T-cell response in LVS-vaccinated individuals (20). It is unknown to what extent these polypeptides are recognized also by T cells from naturally infected individuals. We studied the response of T cells from naturally infected individuals to various polypeptides of LVS membranes. We also asked whether there is heterogeneity among antigen determinants of the four major membrane polypeptides. To this end, T-cell clones were raised against *F. tularensis*.

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MATERIALS AND METHODS

Bacteria and growth conditions. A wild strain of *F. tularensis* biovar palaearctica was isolated in 1981 during an outbreak of tularemia in northern Sweden (13). The attenuated vaccine strain *F. tularensis* LVS was supplied by the U.S. Army Research Institute of Infectious Diseases, Fort Detrick, Md. (4). A capsule-deficient mutant of *F. tularensis* LVS was obtained by acridine orange treatment (19). Bacteria were cultivated for 2 days on modified Thayer-Martin agar containing Gc medium base (21).

Preparation of bacterial membranes. Total membranes and sodium *N*-lauroyl sarcosinate (Sarkosyl [CIBA-GEIGY Corp., Summit, N.J.])-insoluble membranes of *F. tularensis* were prepared as described previously (20).

Analysis and preparation of membrane polypeptides by SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (11). Gradient gels contained 10 to 17.5% acrylamide and 0.20 to 0.46% bisacrylamide. For analytical purposes, total membranes or Sarkosyl-insoluble membranes were suspended in sample buffer (62.5 mM Tris [pH 6.8], 1% SDS, 20% β-mercaptoethanol, 10% glycerol) at a protein concentration of 0.5 mg/ml, estimated as described by Lowry et al. (14). Samples (30 μl) were heated (5 min, 95°C) and subjected to SDS-PAGE. Gels were stained in Coomassie brilliant blue.

For preparative purposes, bacterial membranes of the capsule-deficient mutant of *F. tularensis* LVS were suspended in sample buffer at a protein concentration of 1.0 mg/ml. Samples (100 μl) were heated at 95°C for 5 min and subjected to SDS-PAGE. After overnight electrophoresis, polypeptides were visualized by treating the gels with 0.25 M potassium chloride (6). Membrane fractions I to VII (Fig. 1)

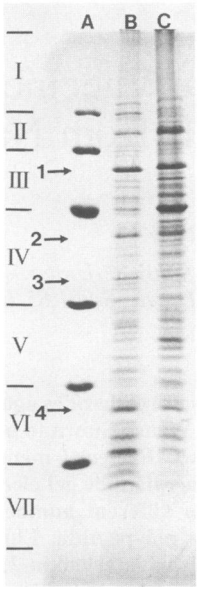

FIG. 1. SDS-PAGE profiles of membranes of *F. tularensis*. Lanes: A, molecular weight markers (phosphorylase *b* [94,000], bovine serum albumin [67,000], ovalbumin [43,000], carbonic anhydrase [30,000], soybean trypsin inhibitor [20,100], and α-lactalbumin [14,400]); B, total membranes of the capsule-deficient mutant; C, total membranes of the wild strain. Roman numbers indicate fractions excised from the gels. Arrows indicate polypeptides excised from gels after SDS-PAGE of Sarkosyl-insoluble membranes.

were excised and subjected to elution. Each fraction contained more than one polypeptide. From separate gels the major polypeptides 1, 2, 3, and 4 (Fig. 1) were excised and eluted (20). When analyzed by SDS-PAGE, each of these eluates was found to contain one major polypeptide. In some eluates, traces of one or a few minor polypeptides were also found. After precipitation with acetone (20), eluted material was resolved in and dialyzed against RPMI 1640 with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (RPMI-HEPES; GIBCO Laboratories, Grand Island, N.Y.).

Assay of proliferative response and IL-2 production of PBMC. Blood samples were obtained from 11 individuals (referred to as no. 1 to 11 in Tables 1 to 3), aged 20 to 60 years, who had undergone tularemia 11 to 23 years before testing. Samples were also obtained from 11 healthy adults (referred to as no. 12 to 22 in Tables 1 and 2) with no previous history of tularemia or tularemia vaccination. Peripheral blood mononuclear cells (PBMC) were prepared from heparinized blood by centrifugation on a Ficoll-Metrizoate (2) gradient (Lymphoprep; NYCOMED AS, Oslo, Norway), and cultures were established as described previously (25). Each culture (200 μl) contained 3×10^5 PBMC. The culture medium consisted of RPMI-HEPES supplemented with 10% pooled human serum, 1% (wt/vol) penicillin, 1% (wt/vol) gentamicin, and 2 mM L-glutamine. As a stimulating agent, membrane polypeptides (10 μg/ml) of *F. tularensis* or purified protein derivative (10 μg/ml) of *Mycobacterium tuberculosis* (Statens Seruminstitut, Copenhagen, Denmark) was used. To estimate the proliferative response, cultures were incubated at 37°C for 6 days, pulsed for 6 h with [^{14}C]thymidine (Amersham International), and harvested (7). To demonstrate the presence of interleukin-2 (IL-2), samples were obtained from the medium of separate cultures after 72 h of incubation and added to concanavalin A-stimulated human PBMC (9). The latter cells were incubated at 37°C for 24 h, pulsed for 24 h with [^{3}H]thymidine (Amersham International), and harvested.

Assay of proliferative response of subpopulations of peripheral blood lymphocytes. Monosized magnetic microspheres (Dynabeads M-450; Dynal AS, Oslo, Norway) were coated by the manufacturer with CD4 or CD8 monoclonal antibodies. $CD4^+$ or $CD8^+$ T cells from [^{14}C]thymidine-incubated PBMC cultures were isolated by positive selection by means of the coated Dynabeads (5). After 5 h of incubation with the isotope, the beads (5.6×10^6 in 40 μl) were added to 1.0 ml of cell culture (containing 1.5×10^6 PBMC). After 30 min of incubation at 4°C with gentle agitation, the beads were removed with a cobalt-samarium magnet. The pellet, containing beads and rosetted lymphocytes, was suspended in chilled saline. To recover rosetted lymphocytes remaining in the supernatant fluid, the procedure was repeated twice. Finally, the radioactivity incorporated in the rosetted lymphocytes was estimated. The procedure yielded 96 to 98% pure preparations, as estimated by immunofluorescence staining by use of Simultest Leu3a/Leu4 (Becton Dickinson and Co., Mountain View, Calif.).

Establishment of T-cell clones. Heparinized blood was obtained from one individual who had been vaccinated with *F. tularensis* LVS 6 years previously. After separation on Lymphoprep, 3.5×10^7 PBMC were mixed in culture medium in 25-ml plastic flasks (Costar, Cambridge, Mass.) with 10^9 heat-killed organisms of the capsule-deficient mutant *F. tularensis* LVS in a final volume of 10 ml. After incubation at 37°C for 6 days, viable cells were recovered on a Lymphoprep gradient, washed, and incubated for an additional 7 days in culture medium, supplemented with 1% (vol/vol) ammonium sulfate-precipitated human buffy coat (BioNative, Umeå, Sweden). The latter supplement equalled 20 U of recombinant IL-2 per ml. Thereafter, cells were cloned by standard procedures (16) by limiting dilution in 96-well microtiter trays (Falcon; Becton Dickinson Labware, Oxnard, Calif.). On average, 0.5 cell was added to each well together with 2×10^4 irradiated (3,000 R) autologous PBMC as feeders, 2×10^7 heat-killed bacteria of the capsule-deficient mutant, and 4 U of IL-2 in 200 μl of culture medium. IL-2 was added every third day. Cells of each culture were washed every 10th day and supplemented with fresh culture medium, 2×10^4 autologous feeder cells, 2×10^7 heat-killed bacteria, and IL-2. When the cell number reached 10^6 per well, cultures were transferred to 24-well plates (Costar) for further expansion of each clone. Expanded clones were recovered on Lymphoprep gradients and tested for antigen-induced proliferation. Each culture contained 2×10^4 cloned cells, 2×10^4 feeder cells, and 2 μg of antigen in 200 μl of culture medium. After incubation at 37°C for 3 days, cells were pulsed for 6 h with [^{3}H]thymidine and harvested. When suitable, clones were saved frozen in liquid nitrogen as well as at −135°C.

RESULTS

Polypeptide patterns of a wild strain of *F. tularensis* and a capsule-deficient mutant of the vaccine strain *F. tularensis* LVS. Total membranes of a wild strain of *F. tularensis* and of the capsule-deficient mutant of *F. tularensis* LVS were subjected to SDS-PAGE. Polypeptide patterns of these strains were very similar, although the intensity of staining of individual proteins varied between the strains (Fig. 1). The four major membrane polypeptides of the mutant, which were used for lymphocyte stimulation (see below), were recognized also in the wild strain (Fig. 1).

TABLE 1. Lymphocyte response to seven fractions containing membrane polypeptides of different molecular weights from *F. tularensis* LVS[a]

Individual no.	Proliferative response (cpm, 10^2) to given fraction[b]								IL-2 production (cpm, 10^2) for given fraction[c]							
	I	II	III	IV	V	VI	VII	Control	I	II	III	IV	V	VI	VII	Control
1	27	31	26	19	25	25	16	1	267	251	239	298	288	278	92	13
2	23	25	22	12	18	25	22	2	374	336	367	354	390	365	264	16
3	6	1	6	2	4	9	10	2	43	40	72	60	74	117	168	15
4	15	12	12	5	11	15	13	3	117	115	198	180	178	192	132	14
5	38	32	28	13	26	34	32	2	316	343	345	335	347	347	286	11
6	29	23	14	9	20	25	24	7	244	233	137	143	278	276	148	43
7	20	14	7	4	9	11	6	2	95	82	66	56	77	69	32	15
8	26	26	33	14	23	21	22	1	115	130	172	192	219	133	64	8
12	1	1	1	1	1	1	1	1	6	6	5	3	3	3	5	2
13	2	4	2	1	2	3	2	1	11	21	5	5	1	1	1	1
14	1	1	1	1	1	1	2	1	10	8	8	5	8	6	11	13
15	2	2	1	1	2	1	1	1	1	2	2	4	6	6	7	3
16	3	4	4	1	2	4	1	2	17	15	15	12	9	12	12	9
17	2	2	3	1	1	1	1	2	18	13	13	11	11	7	9	7

[a] PBMC were obtained from eight individuals (no. 1 to 8) previously undergoing tularemia and from six nonimmunized individuals (no. 12 to 17). Sarkosyl-insoluble membranes of *F. tularensis* LVS were subjected to SDS-PAGE, and gels were separated into seven fractions. Eluates from each fraction were used as antigens (10 μg/ml). Control cultures contained no antigen.

[b] PBMC were pulsed with [^{14}C]thymidine after 6 days of incubation. Means of five cultures are indicated. Standard deviations were <13% of the means.

[c] IL-2 was assayed from samples of culture medium after 72 h of incubation. Means of three to four cultures are indicated. Standard deviations were <17% of the means.

Lymphocyte response to various fractions of membranes from *F. tularensis* LVS. Membranes of the capsule-deficient mutant of *F. tularensis* LVS were separated by SDS-PAGE. After electrophoresis, each gel was divided into seven fractions (Fig. 1). Each fraction was eluted and tested on PBMC from eight individuals previously undergoing tularemia. PBMC from seven of the individuals responded to all and PBMC from one of them responded to some of the fractions with a DNA synthesis higher than that induced in PBMC from any of six nonimmunized individuals (Table 1). Irrespective of fraction, PBMC from all immunized individuals showed an IL-2 production higher than that of PBMC from any of the nonimmunized individuals (Table 1).

Lymphocyte response to major membrane polypeptides of *F. tularensis* LVS. Four major membrane polypeptides of the capsule-deficient mutant of *F. tularensis* LVS were eluted from gels of SDS-PAGE (Fig. 1). Each eluate induced much higher DNA synthesis and much higher IL-2 production in PBMC from naturally infected individuals than in PBMC from nonimmunized individuals (Table 2).

We also estimated the proportion of DNA synthesis occurring in $CD4^+$ and $CD8^+$ T cells of cultures of PBMC from naturally infected individuals. When polypeptide 1, 2, or 3 was used, the incorporation of [^{14}C]thymidine in DNA of $CD4^+$ cells was about three times higher than that in DNA of $CD8^+$ cells (Table 3). With polypeptide 4, the incorporation of $CD4^+$ cells was about double that of $CD8^+$ cells.

Response of T-cell clones to major membrane polypeptides of *F. tularensis* LVS. PBMC from one individual previously vaccinated with *F. tularensis* LVS were cloned by limiting dilution into a total of 600 wells. Nine clones, all $CD4^+$ $CD8^-$, were propagated to the extent that their proliferative responses to the antigens were determined. A clone was considered to respond to an antigen when there were more than 1,000 cpm over background counts and the number of counts was at least twice that of the background (16). By these criteria, all results were reproduced when determinations were repeated at an interval of several weeks. Four clones responded to heat-killed *F. tularensis* but did not respond to any of the four membrane polypeptides (Table 4). One clone (AS-7 in Table 4) showed a vigorous response to all four membrane polypeptides. Clone AS-6 responded to polypeptides 1, 2, and 3, and clone AS-3 responded to polypeptides 2, 3, and 4. Clone AS-2 responded only to polypeptide 1 and clone AS-12 responded only to polypeptide 3. Thus, each of five T-cell clones showed a unique pattern of response to the polypeptides, indicating that at least five distinct determinants occurred among the four

TABLE 2. Lymphocyte response to four major membrane polypeptides of *F. tularensis* LVS[a]

Individual no.	Proliferative response (cpm, 10^2) to given polypeptide[b]					IL-2 production (cpm, 10^2) for given polypeptide[c]				
	1	2	3	4	Control	1	2	3	4	Control
1	5	6	17	19	1	93	82	154	91	18
3	6	8	10	14	1	117	61	104	78	10
4	18	16	26	18	1	90	85	113	65	9
5	25	22	18	18	2	114	82	154	91	16
6	11	5	9	13	1	32	57	50	45	13
7	4	1	2	1	1	66	65	78	66	9
8	15	14	16	28	1	92	124	106	101	10
9	2	2	2	3	1	42	45	49	54	10
10	27	10	8	5	2	100	67	70	41	13
18	1	0	0	1	0	17	21	19	14	13
19	1	1	1	1	1	23	19	17	18	16
20	1	1	1	1	1	19	23	20	21	15
21	2	2	2	2	1	22	18	17	16	15
22	3	2	1	3	0	25	17	16	26	17

[a] PBMC were obtained from nine individuals (no. 1 to 10) previously undergoing tularemia and from five nonimmunized individuals (no. 18 to 22). Sarkosyl-insoluble membranes of *F. tularensis* LVS were separated by SDS-PAGE, and four major polypeptides (10 μg/ml) were eluted from the gels. Control cultures contained no antigen.

[b] PBMC were pulsed with [^{14}C]thymidine after 6 days of incubation. Means of five cultures are indicated. Standard deviations were <15% of the means.

[c] IL-2 was assayed from samples of culture medium after 72 h of incubation. Means of three to four cultures are indicated. Standard deviations were <18% of the means.

TABLE 3. Response of $CD4^+$ and $CD8^+$ T cells to four major membrane polypeptides of *F. tularensis* LVS[a]

Individual no.	T cells	Proliferative response [cpm/10^4 cells (%)[b]] to given polypeptide				
		1	2	3	4	Control
1	$CD4^+$	12.2 (82)	20.2 (92)	18.8 (78)	14.0 (76)	2.6
	$CD8^+$	3.3 (18)	3.1 (8)	6.3 (22)	5.2 (24)	1.6
2	$CD4^+$	16.1 (100)	21.8 (93)	27.1 (96)	24.2 (86)	1.8
	$CD8^+$	1.1 (0)	2.9 (7)	2.3 (4)	4.2 (14)	1.3
4	$CD4^+$	14.3 (79)	9.6 (92)	38.3 (91)	4.0 (47)	0.3
	$CD8^+$	4.8 (21)	1.8 (8)	4.6 (9)	5.1 (53)	1.0
5	$CD4^+$	9.0 (77)	8.4 (65)	7.3 (63)	5.3 (100)	0.4
	$CD8^+$	3.4 (23)	5.1 (35)	4.9 (37)	0.4 (0)	0.8
6	$CD4^+$	22.5 (61)	18.6 (77)	16.4 (90)	3.5 (29)	2.3
	$CD8^+$	14.3 (39)	6.1 (23)	2.8 (10)	4.2 (71)	1.3
7	$CD4^+$	4.3 (100)	3.8 (42)	9.8 (76)	6.1 (56)	0.7
	$CD8^+$	2.0 (0)	6.6 (58)	5.1 (24)	6.6 (44)	2.3
8	$CD4^+$	19.2 (81)	6.0 (82)	12.8 (90)	9.4 (72)	0.6
	$CD8^+$	6.3 (19)	3.2 (18)	3.3 (10)	5.5 (28)	2.0
9	$CD4^+$	22.7 (70)	10.1 (47)	25.4 (70)	15.1 (54)	0.6
	$CD8^+$	10.9 (30)	12.0 (53)	12.2 (30)	13.6 (46)	1.4
11	$CD4^+$	17.2 (98)	3.2 (100)	1.5 (27)	5.3 (100)	0.6
	$CD8^+$	2.3 (2)	2.0 (0)	4.4 (73)	2.0 (0)	2.0
Mean ± SD[c]	$CD4^+$	14.2 ± 5.7	10.2 ± 6.0	16.4 ± 10.6	8.6 ± 6.2	
	$CD8^+$	5.5 ± 4.5	3.2 ± 3.1	3.6 ± 2.8	3.7 ± 3.6	

[a] PBMC from nine individuals previously undergoing tularemia were incubated for 6 days in the presence of antigen (10 μg of membrane polypeptides no. 1 to 4 of *F. tularensis* LVS) and pulsed with [^{14}C]thymidine. $CD4^+$ and $CD8^+$ T cells were isolated by positive selection by means of Dynabeads coated with monoclonal antibodies and assayed for incorporation of radioactivity. Control cultures lacked antigen. Statistical evaluation of differences between results on $CD4^+$ and $CD8^+$ cells: polypeptides 1, 2, and 3, $P < 0.01$; polypeptide 4, $P < 0.10$.

[b] Percentages in each cell fraction after subtraction of background values.

[c] Mean of values from the nine given individuals.

preparations tested. The results also suggested that one determinant occurred on more than one polypeptide.

DISCUSSION

Several membrane polypeptides of the vaccine strain *F. tularensis* LVS were recognized by T cells from individuals previously undergoing tularemia. This implies that there is a strong conformity between the vaccine strain and naturally occurring *F. tularensis* in determinants involved in T-cell recognition. LVS was originally attenuated from a wild strain of *F. tularensis* biovar palaearctica, isolated in the Soviet Union. Although kept in a laboratory for more than 40 years and subjected to repeated passages in antiserum-containing medium (18), polypeptides relevant to the inducement of cell-mediated immunity are well conserved in LVS.

To study the heterogeneity of antigen determinants, we raised T-cell clones against *F. tularensis*. Each of five $CD4^+$

TABLE 4. Proliferative response of T-cell clones to four major membrane polypeptides of *F. tularensis* LVS[a]

T-cell clone	Proliferative response (cpm, 10^3, ± SD)[b] to:					
	Polypeptide				*F. tularensis*	Control
	1	2	3	4		
AS-7	**12.7** ± 1.3	**42.8** ± 1.8	**32.7** ± 3.6	**49.2** ± 3.0	**34.5** ± 3.2	2.1 ± 1.4
AS-6	**2.9** ± 0.5	**13.5** ± 0.4	**5.6** ± 0.2	0.6 ± 4.5	**18.1** ± 0.9	0.4 ± 0.2
AS-3	1.1 ± 0.8	**6.2** ± 1.3	**4.6** ± 1.5	**25.4** ± 10.0	**53.3** ± 12.5	0.4 ± 0.1
AS-2	**7.2** ± 0.5	0.9 ± 0.2	1.1 ± 0.2	0.3 ± 0.1	**9.3** ± 1.9	0.2 ± 0.1
AS-12	1.8 ± 0.5	3.5 ± 1.0	**10.6** ± 0.6	1.6 ± 0.5	**10.8** ± 0.6	3.3 ± 1.0
AS-1	0.4 ± 0.1	0.9 ± 0.4	0.6 ± 0.2	0.8 ± 0.2	**13.5** ± 2.1	0.4 ± 0.1
AS-4	1.3 ± 0.4	1.7 ± 0.3	2.1 ± 0.4	2.4 ± 0.4	**8.3** ± 2.0	1.5 ± 0.4
AS-9	0.8 ± 0.2	0.7 ± 0.2	0.9 ± 0.3	0.7 ± 0.3	**12.3** ± 1.5	0.7 ± 0.3
AS-11	1.1 ± 0.3	1.1 ± 0.3	0.8 ± 0.3	1.2 ± 0.2	**5.3** ± 0.5	1.3 ± 0.4

[a] *F. tularensis*-specific $CD4^+$ T-cell clones were incubated at 37°C for 3 days in the presence of membrane polypeptides 1 to 4 (10 μg/ml) of *F. tularensis*, heat-killed *F. tularensis* (10^8/ml), or without antigen (control) and pulsed with [^{3}H]thymidine.

[b] Means of five cultures. Boldfaced values indicate responses >1,000 cpm over background counts and number of counts at least twice that of the background.

$CD8^-$ clones showed a unique pattern of response when tested with four major membrane polypeptides. Moreover, there were clones which did not recognize any of the four polypeptides but still recognized *F. tularensis*. These data together with the finding that seven fractions of the bacterial membranes all stimulated T cells from immunized individuals indicate that the total number of different T-cell-reactive determinants of *F. tularensis* may indeed be high. Heterogeneity of T-cell-reactive determinants has been observed in other facultative intracellular bacteria. Mycobacteria (12, 16, 17) and *Listeria monocytogenes* (27) seem to possess several T-cell-reactive determinants. By use of T-cell clones, four different antigen determinants were recognized on a 36-kilodalton polypeptide of *Mycobacterium leprae*, and one of these determinants occurred also on a 12-kilodalton polypeptide of the species (17). The ability to recognize a multitude of determinants may be important to the host defense against facultative intracellular bacteria. Such a principle would be quite different from that involved in the induction of antibody-mediated defense against many extracellular bacteria, a situation in which immunization with one single antigen can be sufficient to afford protective immunity.

F. tularensis may be useful in studies on the possible importance of a diverse T-cell response in infections with intracellular bacteria. First, we showed that the organism possesses several strongly immunogenic polypeptides, as evidenced by their ability to induce a T-cell response in most naturally infected individuals. It should be remarked that these individuals had contracted tularemia 11 to 23 years before our testing. Second, the immune response to this wide array of polypeptides was highly specific. T cells from nonimmunized individuals responded poorly to all seven membrane fractions as well as to all four major polypeptides tested. This indicates that inadvertent immunization towards *F. tularensis* due to cross-reacting microorganisms is unusual. Still, the complete lack of response of the nonimmunized individuals to all preparations is remarkable. Many clinically important bacteria are known to be mitogenic to human lymphocytes; i.e., they stimulate the lymphocytes without regard to previous sensitization. This is true also for facultative intracellular bacteria, such as mycobacteria (1) and *L. monocytogenes* (27). The present use of a capsule-deficient mutant of *F. tularensis* for preparation of membrane antigens may have contributed to give a high immunological specificity.

A final elucidation of the diversity of T-cell-reactive determinants of *F. tularensis* will probably require sequence determination of membrane polypeptides of the species. This will now become possible, since structural genes of *F. tularensis* polypeptides have been cloned and expressed in *Escherichia coli* (15, 24). One of the membrane polypeptides (no. 4 in Fig. 1) has been expressed by *E. coli* in a form allowing immunospecific T-cell stimulation (24). The cloning will enable determination of nucleotide sequences, deduction and synthesis of suitable peptide sequences, and probing of the sequences for T-cell recognition.

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