Kinetics of the Immune Response Associated with Tularemia: Comparison of an Enzyme-Linked Immunosorbent Assay, a Tube Agglutination Test, and a Novel Whole-Blood Lymphocyte Stimulation Test

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We have developed and evaluated a novel and simplified whole-blood lymphocyte stimulation assay that focuses on the measurement of gamma interferon after 24 h of stimulation with whole-cell tularemia antigen and a tularemia enzyme-linked immunosorbent assay (ELISA) based on highly purified lipopolysaccharide antigen. Comparison of the kinetics of the two assays and those of the traditional tube agglutination test shows that the cellular immune response can be detected earlier by the lymphocyte stimulation assay. This test already shows a high proportion of positive results during the first week after the onset of the disease, may be applicable in everyday laboratory practice, and has the potential of changing routine diagnostics for tularemia.

The new ELISA has a high sensitivity and becomes positive to a high degree during the second week of disease.

The zoonotic disease tularemia, caused by the fastidious gram-negative rod \textit{Francisella tularensis}, can appear in various clinical forms, depending on the mode of transmission of the disease (4). In most areas where it appears, whether it is endemic or emerging, the disease is only rarely seen. In some cases, e.g., typical ulceroglandular cases, a clinical diagnosis can be made with a high degree of certainty. In other cases, e.g., typhoidal and respiratory cases, a clinical diagnosis of tularemia is almost impossible to make with enough certainty. Because it is a rare disease and has a multitude of clinical presentations, patients with the disease present in various ways. Thus, because it seldom appears and has various forms, the suspicion of tularemia can be raised in different clinical settings and medical specialties. Since the treatment of tularemia differs from the treatment of many other diseases with a similar clinical appearance, confirmation of the diagnosis is of utmost importance. Laboratory confirmation of the diagnosis can be based on culture, PCR methods, or antibody detection. Culture of \textit{F. tularensis} is usually avoided because of the risk of spread of the pathogen to laboratory personnel (20). The detection of \textit{F. tularensis} by PCR in samples from primary lesions in patients with ulceroglandular tularemia is a fast and sensitive way of confirming the diagnosis, but it is not applicable to the detection of \textit{F. tularensis} in cases in which the diagnosis is the most difficult to make, i.e., typhoidal and respiratory cases (7). Antibody analyses, both agglutination methods and established enzyme-linked immunosorbent assay (ELISA) methods, have relatively high sensitivities and specificities (3, 10, 15, 17, 22). Unfortunately, however, the serologic tests described often do not become positive until the third week of disease (24, 28). Recently, Western blotting has been shown to be useful as a confirmation test in areas where tularemia is rare (21).

In the county of Örebro, situated in central Sweden, tularemia has emerged since the year 2000, with 337 cases reported between 2000 and 2006, while only 11 cases had been reported between 1980 and 1999 (5, 6). An overwhelming majority of the patients have been treated at the Department of Infectious Diseases, Örebro University Hospital. Even though patients have been cared for by doctors familiar with tularemia, diagnosis of the disease has proved to be a challenge. Accordingly, there is a need for faster diagnostic tools for confirmation of the diagnosis of tularemia. Cellular immunity, measured by lymphocyte stimulation tests, develops earlier in the course of the disease than measurable antibody production (15, 26). Additionally, since \textit{Francisella} grows intracellularly, an efficient immune response to tularemia is dependent on a strong cell-mediated component, and patients with tularemia with negative serology results but positive lymphocyte stimulation test results have been described (12, 19). Traditional lymphocyte stimulation methods are, however, laborious, time-consuming, and not suitable for routine use in clinical practice (26). Recently, simplified methods based on the stimulation of lymphocytes in whole blood, e.g., the flow cytometric assay of the specific cell-mediated immune response in activated whole blood (FASClIA), have been developed (11, 25). We have adapted this method by concentrating on the release of cytokines from stimulated lymphocytes. We have also established an ELISA based on highly purified \textit{F. tularensis} lipopolysaccharide (LPS) for the detection of immunoglobulin G (IgG) and IgM antibodies to \textit{F. tularensis}.

The aim of this study was to evaluate the usefulness of the
new lymphocyte stimulation test and the ELISA compared to that of the traditional tube agglutination test for the early diagnosis of tularemia.

**MATERIALS AND METHODS**

**Study subjects.** Blood samples from 20 healthy blood donors (Table 1, group A) were used to establish the cutoff levels for the lymphocyte stimulation test.

Nineteen subjects (Table 1, group B) who had an acute febrile condition and in whom tularemia was suspected were sampled two times per week for 2 to 3 weeks. Tularemia was later confirmed in 14 of the subjects by a fourfold rise in antibody titers by the tube agglutination test method (n = 13), a positive culture result (n = 4), and/or a positive result by PCR analysis performed with a sample from the primary lesion (n = 4). In five subjects, tularemia could be ruled out because their convalescent-phase sera had negative agglutination titers and, in some of the cases, because of a definitive diagnosis other than tularemia. Additionally, samples were obtained from two subjects (Table 1, group C) with other acute febrile conditions; however, the sample were not obtained consecutively.

For the F. tularensis ELISA, the cutoff values for IgG and IgM were calculated after the analysis of sera from 50 tularemia-negative subjects (Table 1, group D). Of these 50 subjects, 29 were sampled because of a clinical suspicion of tularemia, but acute-phase and convalescent-phase sera from these subjects were negative for F. tularensis by the tube agglutination test. Convalescent-phase sera from these 29 subjects were used. Additionally, sera from 16 persons negative for F. tularensis by the tube agglutination test and positive by streptococcal serology as well as convalescent-phase sera from the 5 tularemia-negative subjects included in the lymphocyte stimulation mentioned above were analyzed.

To study the kinetics of the ELISA, consecutive samples from 24 subjects (Table 1, group E) were analyzed. Of these subjects, the samples from 14 subjects were included in the lymphocyte stimulation part of the study, while consecutive samples from 10 other subjects were available for antibody analyses only.

The persistence of antibodies in 24 other subjects (Table 1, group F) from whom sera were obtained 14 to 54 months after their episode of tularemia (i.e., late-phase sera) was studied by IgG ELISA, IgM ELISA, and the tube agglutination assay. Among the subjects providing late-phase sera, the diagnosis of tularemia had previously been confirmed in 8 subjects by culture and/or PCR analysis, while tularemia was confirmed in the remaining 16 subjects by the tube agglutination assay. Of these 16 subjects, 4 had a fourfold increase in antibody titers and the other 12 had a titer of at least 1/80.

Finally, 9 of the 14 subjects with confirmed tularemia (Table 1, group B) were resampled approximately 2 years after their tularemia episodes. Lymphocyte stimulation was performed with these samples within a few hours of collection but also after 24 h, in which the samples were sent to the laboratory through the internal hospital postal service, and after 48 h, in which the samples were sent to the laboratory by ordinary mail, thus imitating the normal procedure for referred samples.

**ELISA for detection of F. tularensis antibodies.** Commercial ELISA plates (Immunoplate MaxiSorp; Nunc) were coated with a highly purified LPS from the Francisella tularensis live vaccine strain (31), kindly provided by Wayne Conlan, NRC, Ottawa, Ontario, Canada. Before the plates were coated, the antigen was diluted to a concentration of 3 μg/ml. A coating buffer was used as the diluent and contained 3.47 g NaHCO₃, 1.18 g Na₂CO₃, 0.2 g NaN₃, and ultrapurified water to 1,000 ml. After the antigen was added, the plates were kept overnight at room temperature. The excess antigen was then removed and 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) was added to the wells, after which the plates were stored in a refrigerator until use. Before use, the wells were washed with washing buffer (0.9% NaCl, 0.05% Tween 20 [Merck], 0.05% BSA). The sera from the subjects were diluted 1/1,000 in incubation buffer (0.2 g NaNO₂ and 0.5 ml Tween 20 in 1,000 ml PBS) and added to the wells. A total of 100 μl of each diluted serum sample was added to each of four antigen-coated wells and to two wells coated with buffer only. After 3 to 5 h of incubation at room temperature, the plates were washed as described above, and alkaline phosphatase-conjugated rabbit anti-human IgG and IgM (Dako Cytomation) were added, after which the plates were kept at room temperature overnight. On the following day, the plates were washed as described above. Substrate was produced by adding a substrate tablet (104 phosphatase substrate; Sigma) to 5 ml of substrate solution (97 ml diethanolamine, 0.101 g MgCl₂ · 6H₂O, 0.2 g NaN₃, and ultrapurified water to 1,000 ml; the pH was adjusted to 9.8 with 1 M HCl). A total of 100 μl substrate solution was added to each well. The absorbance was read at 405 nm. Reading of the reaction was done when the value for the calibration serum samples reached 1.0. To obtain the final ELISA titers, the results were multiplied by the dilution of the sera. The mean value for IgG and IgM, respectively, for samples from group D (Table 1) plus 2 standard deviations was used as the cutoff level.

**Lymphocyte stimulation test.** Lymphocyte stimulation was performed by using a modification of a simplified whole-blood protocol, FASCIA, which was described recently (11, 25). Peripheral blood was drawn from the subjects and placed into heparin-containing vacuum tubes (Venoject VT-100SH; Terumo Europe N.V., Belgium), kept at room temperature, and processed within a few hours. Each sampling strategy was also obtained and kept at −80°C until antibody analyses were performed. For lymphocyte stimulation, 1 ml whole blood was diluted with 9 ml RPMI 1640 with glutamine (GIBCO Invitrogen, United Kingdom) supplemented with 10 μg/ml gentamicin (GIBCO Invitrogen, United Kingdom). A commercial whole-cell preparation based on formalin-killed live vaccine strain bacteria (Reagensia AB, Stockholm, Sweden) was used as the tularemia antigen. The antigen was diluted 1/100 in dilution medium. Phytohemagglutinin (Sigma-Aldrich) at a final concentration of 5 μg/ml was used as a positive control. Medium only was used as a negative control. A total of 200 μl each of diluted antigen (in triplicate), the positive control, and the negative control was distributed into sterile Falcon 2058 test tubes (BD Biosciences). A total of 1,800 μl of diluted blood was added to each test tube, and the tubes were then incubated in a humidified atmosphere at 37°C with 5% CO₂ in air. After 24, 48, and 72 h, the contents of each tube were gently mixed, and 500 μl was removed and centrifuged at 3,000 × g for 5 min. The supernatant was removed and kept at ~80°C until the cytokine analyses were performed. In the initial experiments, the samples were stimulated for a maximum of 7 days, according to the protocol described by Gaines and colleagues (11, 25). However, in our system, a prolonged incubation only added higher background levels and less specific reactivities (data not shown). The concentration of gamma interferon (IFN-γ), which was used as a measure of the lymphocyte stimulation, was determined by using a commercial ELISA kit (human IFN-γ Quantikine ELISA; R&D Systems Europe Ltd.). The choice of IFN-γ was made after pilot studies with a human Th1/Th2 cytokine kit (BD Biosciences) that also measured the concentrations of tumor necrosis factor alpha, interleukin 2 (IL-2), IL-4, IL-6, and IL-10. In initial experiments, lymphoblast formation was measured by flow cytometry (Altra; Beckman Coulter) as a proxy for lymphocyte proliferation (11). A high correla-

| Group | Use                          | No. of samples | Type of subjects          |
|-------|------------------------------|----------------|----------------------------|
| A     | Cutoff levels in lymphocyte stimulation | 20             | Healthy blood donors       |
| B     | Lymphocyte stimulation, consecutive samples | 19             | 14 subjects in whom tularemia was confirmed, 5 subjects in whom tularemia was ruled out |
| C     | Lymphocyte stimulation, acute- and convalescent-phase samples | 2             | 2 subjects in whom tularemia was ruled out |
| D     | Cutoff levels for IgG and IgM ELISAs | 50             | 29 subjects in whom tularemia was suspected but ruled out, 16 subjects with streptococcal infection, 5 subjects from group B in whom tularemia was ruled out |
| E     | ELISA, consecutive samples | 24             | 14 subjects from group B in whom tularemia was confirmed, 10 other subjects in whom tularemia was confirmed |
| F     | Persistence of antibody titers | 24             | Late-phase samples from subjects in whom tularemia had been confirmed earlier |

**TABLE 1. Use of samples from study subjects**

| Group Use No. of samples | Type of subjects |
|-------------------------|-----------------|
| Group A                 | Healthy blood donors       |
| Group B                 | 14 subjects in whom tularemia was confirmed, 5 subjects in whom tularemia was ruled out |
| Group C                 | 2 subjects in whom tularemia was ruled out |
| Group D                 | 29 subjects in whom tularemia was suspected but ruled out, 16 subjects with streptococcal infection, 5 subjects from group B in whom tularemia was ruled out |
| Group E                 | 14 subjects from group B in whom tularemia was confirmed, 10 other subjects in whom tularemia was confirmed |
| Group F                 | Late-phase samples from subjects in whom tularemia had been confirmed earlier |

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tion between the level of lymphoblast formation and the level of IFN-γ secretion in the supernatants was found. The levels of the other cytokines studied did not correlate well with the level of lymphoblast formation (data not shown). The cutoff level was calculated by using the mean IFN-γ value in the samples from 20 healthy blood donors (Table 1, group A) plus 2 standard deviations.

Tube agglutination. Tube agglutination was performed according to the classical Widal reaction, as described previously (10, 22), by using the commercial whole-cell preparation mentioned above. This was the accredited routine diagnostic method at the Örebro University Hospital at the time of the study. A titer of 1/40 or above was regarded as a positive result.

RESULTS

ELISA. The cutoff levels for IgG and IgM were calculated to 160 arbitrary units (range for the subjects, 0 to 293 arbitrary units, with two subjects having levels above the cutoff) and 120 arbitrary units (range, 0 to 254 arbitrary units, with two subjects having levels above the cutoff), respectively, for samples from group D (Table 1).

For the 24 subjects from whom consecutive serum samples were obtained during the acute phase of tularemia (Table 1, group E), the results are presented as the cumulative proportion of sera positive by the IgG ELISA, the IgM ELISA, the IgG and/or IgM ELISA, and the tube agglutination test (Fig. 1). Additional sera obtained at later times showed seroconversion in some subjects, giving final sensitivities of 92, 100, and 100% for the IgG, IgM, and IgG and/or IgM ELISAs, respectively (Table 2).

For the 24 subjects studied for evaluation of the persistence of antibodies (Table 1, group F), the proportion of subjects seropositive by the different analyses at late sampling times is shown in Table 3. Despite the similar proportions of sera obtained at late convalescent phase that were positive for IgG and IgM, the IgG/IgM ratio was found to be rising in all 13 subjects when the values for sera that were taken more than 3 weeks but less than 1 year after the onset of the disease could be compared with the values for late-phase sera (taken 14 to 54 months after the onset of disease).

Lymphocyte stimulation. The cutoff level for the IFN-γ concentration after 24 h of stimulation for the samples from the 20

![Fig. 1. Cumulative proportion of subjects with significantly reactive serological assay results (24 subjects with tularemia) and cellular immune responses measured by the novel lymphocyte stimulation test (14 of 24 subjects with tularemia).](http://cvi.asm.org/)

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**TABLE 2. Sensitivities of the ELISAs**

| Time of assay and ELISA type | No. of samples positive/total no. of samples tested | % Sensitivity |
|-----------------------------|---------------------------------|--------------|
| 3 wk after disease onset    |                                 |              |
| IgG                         | 21/24                           | 88           |
| IgM                         | 21/24                           | 88           |
| IgG and/or IgM              | 23/24                           | 96           |
| Within two months           |                                 |              |
| IgG                         | 22/24                           | 92           |
| IgM                         | 24/24                           | 100          |
| IgG and/or IgM              | 24/24                           | 100          |

**TABLE 3. Proportion of subjects with tularemia with significantly reactive tests determined with samples drawn 14 to 54 months after acute tularemia**

| Analysis                      | No. of subjects with significantly reactive test results/total no. of subjects | % Sensitivity |
|-------------------------------|-----------------------------------------------------------------------------|--------------|
| IgG ELISA                     | 21/24                                                                       | 87.5         |
| IgM ELISA                     | 22/24                                                                       | 91.7         |
| IgG and/or IgM ELISA          | 24/24                                                                       | 100          |
| Tube agglutination            | 17/24                                                                       | 70.8         |
healthy blood donors (range, <8 to 110 pg/ml, with 1 of the 20 subjects having a level above the cutoff) was calculated to be 82 pg/ml (Table 1, group A). The initial samples were stimulated for 72 h, but the supernatants were collected after 24, 48, and 72 h. For samples collected at later times, stimulation was restricted to 48 h since a preliminary analysis of the data showed that the longest stimulation time did not add any further information. At the final analysis of the data, even 48 h of stimulation did not add any further information compared to the information obtained at 24 h of stimulation (data not shown); thus, the data obtained at 24 h were used.

Lymphocyte stimulation was performed with samples from 21 subjects. Consecutive blood samples were obtained from 19 subjects (Table 1, group B), 14 of whom had tularemia and 5 of whom did not have tularemia, but consecutive samples were not available from 2 other subjects who did not have tularemia (Table 1, group C).

Samples from all 14 subjects with tularemia showed IFN-γ levels clearly above the cutoff value (mean of the highest levels, 1,412 pg/ml; range, 267 to 4,354 pg/ml) after stimulation. This was already achieved either for the first sample obtained, as was the case for 10 of the subjects, or during the course of the sampling period, as was the case for the other 4 subjects. Evidence of seroconversion, as determined by the agglutination assay, and/or a positive culture result was obtained for 9 of the 10 subjects whose first sample was already positive by the lymphocyte stimulation test, excluding the possibility that a previous episode of tularemia was the cause of the lymphocyte stimulation result.

The IFN-γ levels in blood samples from six of seven subjects who did not have tularemia were consistently below 20 pg/ml after stimulation. The samples from one of the subjects who did not have tularemia, however, developed rising levels of IFN-γ, which peaked at 813 pg/ml. This sample was from a 35-year-old man who was negative for tularemia by all serological analyses, had lymphocytosis and atypical lymphocytes, and had a primary cytomegalovirus (CMV) infection that was confirmed serologically.

The antibody levels in consecutive serum samples were also analyzed by both ELISA and the tube agglutination method, as described above. The results of the serological methods, as well as those of the lymphocyte stimulation assay, are presented as the cumulative proportion of subjects with significantly reactive tests and are shown in Fig. 1. Fifty percent of the subjects had significantly reactive lymphocyte stimulation test results 6 days after the onset of the disease, while the same proportion of seropositive subjects was noted after 13 days and 14 days for the IgM and IgG ELISAs, respectively. If the criterion was positivity by the IgM and/or IgG ELISA, 50% of the subjects were seropositive after 10 days. The same proportion of subjects were positive by the agglutination method 15 days after the onset of the disease.

The lymphocyte stimulation tests performed with samples taken from 9 of the 14 subjects 2 years after their documented episode of tularemia showed markedly lower levels of IFN-γ (Table 4).

Among the samples stimulated after routine transportation for 24 h and 48 h, the samples stimulated with tularemia antigen (Table 4) as well as the positive controls showed insignificant levels of IFN-γ.

Table 4. IFN-γ levels in samples from nine tularemia subjects after lymphocyte stimulation

| Subject no. | Peak in 2005 | In last sample tested in 2005 | In samples obtained in 2007 and stimulated: |
|-------------|-------------|-----------------------------|---------------------------------------------|
|             | Immediately | After a 24-h delay | After a 48-h delay |
| 3           | 1,520       | 1,520          | ND*          | ND          | ND          |
| 4           | 820         | 820            | 215          | 34          | 8           |
| 5           | 265         | 180            | 110          | 16          | 8           |
| 6           | 405         | 375            | 89           | 8           | 8           |
| 8           | 1,960       | 1,960          | 415          | 110         | 20          |
| 9           | 2,850       | 2,850          | ND           | ND          | ND          |
| 10          | 1,720       | 1,720          | 9            | 8           | 8           |
| 12          | 1,360       | 820            | ND           | ND          | ND          |
| 13          | 305         | 65             | 8            | 8           | 8           |
| 14          | 225         | 195            | ND           | ND          | ND          |
| 17          | 4,350       | 955            | ND           | ND          | ND          |
| 18          | 535         | 260            | 115          | 8           | 8           |
| 19          | 1,470       | 1,470          | 130          | 20          | 8           |
| 20          | 1,940       | 990            | 275          | 54          | 8           |

* ND, not done.

DISCUSSION

The need for better diagnostic tools for confirmation of a diagnosis of tularemia earlier during the clinical course of infection is of importance, as was already argued for above. In an earlier study, we were able to show that during the first 3 years after the emergence of tularemia in central Sweden, as many as 35 of 62 patients were treated with antibiotics inappropriately for the disease (5). We could also demonstrate that patients with glandular complications, such as spontaneous suppuration, had had a significantly longer delay from the time of onset of the disease to the time of initiation of adequate antibiotic treatment than patients without complications.

The need for better tools for the early confirmation of a diagnosis of tularemia in patients prompted this investigation of the different diagnostic methods applicable in clinical practice.

Our tularemia ELISA with highly purified Francisella LPS as the antigen showed results similar to those of previously described ELISAs, with the results becoming positive for a high proportion of the subjects during the second week of the disease (3, 17, 27). If the definition of a positive ELISA result is when the values of the IgG and/or IgM ELISAs are above the cutoff, the ELISA became positive for 50% of the subjects after 10 days, 5 days before the agglutination test had the same proportion of positive results.

There was no obvious relationship between the levels of IgG and IgM reached, as the IgG levels were often high when the IgM levels were low and vice versa. All test results for subjects with confirmed tularemia, however, became positive by the IgG and/or IgM ELISA within 25 days after the onset of the disease. By the tube agglutination method, on the contrary, the results for 20% of the samples were still negative after 3 weeks of disease, even though a fairly low titer of 1/40 was used as the level for seropositivity. One subject became positive by the IgM ELISA on day 25 but was still negative by the IgG ELISA and the agglutination test on the same day, after which...
further samples were not obtained. This subject had a typical ulceroglandular tularemia, and the diagnosis was confirmed by a positive PCR test result with a sample from the primary lesion. It is possible that this subject would have become seropositive by other antibody tests if samples had been obtained at later times. There are, however, as mentioned before, reports of patients with confirmed tularemia with sustained negative responses by antibody tests (12). Regardless, a late seroconversion is of little use in the clinical setting, where the treatment decision must be made as early as possible when tularemia is suspected.

Late-phase sera were, for most of our subjects, obtained 14 to 20 months after their disease episodes, and samples were obtained after more than 3 years from only a few subjects. The rates of persistence of both IgG and IgM antibodies were high, as measured by the ELISA method. As has been shown before, tularemia differs in this aspect from most other infections (28). It has been suggested that the IgG/IgM ratio be used as a tool for differentiating between a recent and an older infection (1, 3). Accordingly, the IgG/IgM ratio was higher in late-phase sera than in sera obtained earlier for any given subject, suggesting a more rapid decline in IgM levels than in IgG levels. Because of the overlapping of ratios for late-phase sera and sera obtained earlier between different subjects, our findings do not support the use of the IgG/IgM ratio for differentiating between an older and a recent infection in a patient on the basis of an analysis of a single serum sample. The results of the ELISA showed a higher proportion of significantly reactive test results than the proportion obtained by the agglutination tests with late-phase sera.

In humans, tularemia is followed by a long-lasting cellular immune response, as can be demonstrated by lymphocyte stimulation tests or by delayed-type hypersensitivity testing by the intracutaneous injections of tularemia antigen (2, 9, 18, 30). The cellular response mainly appears through a Th1-like activation of T lymphocytes, which produces tumor necrosis factor alpha, IL-2, and IFN-γ. In a previous study, the cellular response was shown to be directed mostly against proteins, while the humoral response targeted carbohydrate structures (23). No correlation between the degree of the cellular response and antibody levels was observed in previous studies (16, 29, 30).

As mentioned above, the cellular immune response can be demonstrated earlier than antibody production during the course of the disease. The tularemia skin test has been shown to be positive as early as 4 days after the onset of the disease, with 29% of patients being positive after 1 week of disease and 95% being positive after 2 weeks of disease (2, 9). Problems with standardization of the skin test have, however, occurred, and it is rarely used nowadays (18).

The lymphocyte stimulation test has been suggested to be a tool for the diagnosis of tularemia (26, 30). Its use as a diagnostic method in clinical practice has been limited, however, because of the usual 1-week stimulation period of traditional lymphocyte stimulation methods and because the analysis is laborious. However, simplified and more rapid methods for lymphocyte stimulation for the detection of immunity to tularemia have been published, including analyses of the cytokines IL-2 and IFN-γ (13, 14). We chose to adapt the recently documented FASCIA method for lymphocyte stimulation, using the level of IFN-γ in the supernatant as a measure of the degree of activation.

When this simplified whole-blood lymphocyte stimulation test was compared with the ELISA and the agglutination test, a positive response appeared much earlier, as has been shown before by established methods that stimulate the lymphocytes for a longer time. In our study, stimulation for 24 h was sufficient and resulted in a 50% cumulative proportion of subjects already having significantly reactive test results after 6 days of disease, and all 14 subjects with tularemia were positive within 12 days. The results may be better, since the first blood samples from 10 of 14 subjects were already positive, with the limiting factor for these subjects being the patients’ delay in seeking treatment.

The samples from 1 of the 14 subjects with tularemia showed a positive but weak response, with the IFN-γ concentration reaching 307 pg/ml 8 days after the patient fell ill, while the levels dropped later. This subject, an 84-year-old man with diabetes mellitus, had a documented episode of herpes zoster whose onset was 7 days after he fell ill with tularemia. It can be debated whether the cellular immunity against tularemia was suppressed by his herpes zoster or if the lower level of response in the lymphocyte stimulation test as well as the herpes zoster infection was due to an already existing immunodeficiency. The subject showed seroconversion against tularemia by both the ELISA and the agglutination test.

As to the specificity of this method of lymphocyte stimulation, six of seven controls with febrile conditions other than tularemia showed little or no response after stimulation. The samples from one control subject with a primary CMV infection, however, showed a rising level of IFN-γ over time, reaching a peak at over 800 pg/ml, 10 times the cutoff level. This could represent cross-reactivity between antigens from the two microorganisms but could also be due to other mechanisms. A convalescent-phase sample from a control subject who also had a primary CMV infection was negative by the lymphocyte stimulation test. Further studies with subjects with febrile conditions other than tularemia, including CMV and Epstein-Barr virus infections, are needed.

The markedly lower levels of IFN-γ obtained after 24 h of stimulation of samples obtained from subjects 2 years after their episode of tularemia compared to the levels achieved during the tularemia episodes is somewhat surprising and is contrary to the results of conventional lymphocyte stimulation tests performed with samples from subjects 25 years after their tularemia infections (8). Our results suggest that this new whole-blood lymphocyte stimulation method could be of value in differentiating between recent and older infections. This is of particular interest in cases of suspected tularemia in areas where the disease is endemic. Further studies are, however, needed on this matter. Insignificant levels of IFN-γ were achieved in two of our subjects, one of whom was the subject who concomitantly had tularemia and a herpes zoster infection (Table 4, subject 13).

A limiting value of the lymphocyte stimulation method described here is the need for processing of the samples within a few hours, since a delay of 24 h or more seems to impair the results. Further studies of this are needed as well to find methods less sensitive to a delay in time to enhance the applicability of the analysis in routine diagnostics.
In conclusion, a tularemia-specific ELISA, based on highly purified LPS, shows high sensitivity, and the results usually become positive during the second week of disease. A simplified whole-blood method for measurement of the cellular immune response looks to be even more clinically useful, as samples from more than 50% of the subjects were already positive during the first week of disease. The method seems to be well suited for routine use with samples from patients with suspected tularemia if methods that allow overnight transportation of samples without compromising the results can be developed. As pointed out here, further research is also needed on other aspects, including analyses of samples from subjects with tularemia as well as from subjects with other infections, to study the specificity of the assay and the long-term persistence of a demonstrable immune response.

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