JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1979, p. 854-860
0095-1137/79/12-0854/07$02.00/0

Vol. 10, No. 6

Time of Lymphocyte Response After Onset of Tularemia and After Tularemia Vaccination

A. TÄRNVIK,* G. SANDSTRÖM, AND S. LÖFGREN

Department of Clinical Bacteriology, University of Umeå, Sweden

Received for publication 8 May 1979

Blood lymphocytes were prepared from 6 patients at various time intervals after the onset of tularemia and from 10 subjects after vaccination against this disease. Lymphocytes were also prepared from subjects who had been vaccinated 1 and 2 years previously. The lymphocytes were incubated in the presence of membranes of the vaccine strain. Lymphocytes obtained 2 weeks or later after onset of the disease responded to the membranes with increased deoxyribonucleic acid synthesis, whereas lymphocytes obtained earlier than 2 weeks after onset did not respond. Lymphocytes of the vaccinated subjects did not respond to the membranes of the vaccine strain before vaccination. Two to 4 weeks after vaccination lymphocytes from six of the vaccinees yielded a high response, and this response was consistently high for several months. Lymphocytes from four of the vaccinated individuals responded to a low extent only, and this was consistently low for several months. Lymphocytes from individuals vaccinated 1 year before testing responded to a similar extent to the membranes, as did lymphocytes from those who had been vaccinated 1 month previously. Lymphocytes from individuals vaccinated 2 years previously, however, showed a diminished response to the membranes. There was no correlation between titer of agglutinating antibodies and magnitude of lymphocyte reactivity.

The lymphocyte stimulation test has been used to demonstrate an immune response in humans to *Francisella tularensis* (17, 18). In this test, the stimulation of blood lymphocytes by antigenic preparations of a vaccine strain, *F. tularensis* LVS, was assayed by incorporation of radioactively labeled thymidine into deoxyribonucleic acid (DNA) of the lymphocytes. Lymphocytes from vaccinated individuals and from tularemia patients responded to the antigenic preparations with an increased DNA synthesis, whereas lymphocytes from unexposed individuals responded poorly or not at all. Most of the responding cells were found to be T lymphocytes, and a minor fraction were B lymphocytes (18). Thus, the lymphocyte stimulation test seems to estimate cell-mediated rather than humoral immunity to *F. tularensis*.

Cell-mediated immunity is known to correlate better than humoral immunity with host resistance against various intracellular bacteria including *F. tularensis* (1, 7, 10, 13, 15, 19). Therefore, the lymphocyte stimulation test may become an in vitro assay of resistance against *F. tularensis*.

In the present study, the time of appearance of lymphocyte response to *F. tularensis* antigen after the onset of tularemia and after tularemia vaccination was studied. The response of lymphocytes from individuals vaccinated 1 and 2 years previously was also studied.

MATERIALS AND METHODS

Tularemia patients. Six tularemia patients were included in the study. They lived within an area of about 50 km^2 in the county of Västerbotten in northern Sweden and became ill during the first part of August in 1978. They had all been in the woods within a week before onset of the disease. When first visited by one of us (A.T.), each of five patients had a rounded red efflorescence about 1 cm^2 in size with a central pustule or ulcer. The efflorescence was located on the neck, arm, or leg and did not bother the patient. The other patient became ill 13 days before our visit. She reported a similar efflorescence. Each patient had a clearly palpable regional lymphadenit. The onset of fever varied from 4 days before to 6 days after the patients first noticed their efflorescence. All patients received doxycycline, 200 mg on day 1 of treatment and 100 mg on each of the following 9 days. Treatment was initiated within 4 days of onset of fever in five cases. They responded promptly to treatment and became afebrile within a week. The sixth patient did not get doxycycline until day 11 after the onset of fever. She was still febrile 14 days after initiation of treatment. She then received another course of treatment with a double dose of doxycycline. She recovered after that.

Venous blood was obtained from the patients at various time intervals for three weeks after the onset

of disease and put into heparinized, and nontreated glass tubes (100 by 16 mm; Becton, Dickinson & Co., Rutherford, N.J.).

Vaccinated individuals. Ten healthy individuals were vaccinated with *F. tularensis* LVS, which was supplied lyophilized by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md. Before and at various intervals after vaccination, samples of venous blood were obtained and put in heparinized and nontreated tubes. Fourteen and 18 other healthy individuals had been vaccinated 1 and 2 years, respectively, before sampling. All individuals were vaccinated intradermally by the same vaccinator, using vaccine from one batch.

Lymphocyte-stimulating agents. Membranes of *F. tularensis* LVS were prepared in a similar way to that used to isolate outer membranes of *Escherichia coli* and *Neisseria gonorrhoeae* (18, 20, 21). Purified protein derivative of tuberculin (Statens Seruminstitut, Copenhagen, Denmark) was also used. The membranes were suspended and the purified protein derivative was solubilized in RPMI 1640 *N*-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid containing 100 μg of streptomycin sulfate per ml (RPMI-HEPES).

Serum. Venous blood from tularemia patients and vaccinated individuals was centrifuged. Serum was inactivated at 56°C for 30 min and kept frozen at −20°C until testing. Serum was also pooled from several healthy blood donors, inactivated, and kept frozen.

Lymphocyte cultures. Lymphocytes were prepared from heparinized blood by centrifugation on Lymphoprep (Nyegaard A/S, Oslo, Norway) as described by Böyum (4), washed, and suspended in a culture medium containing RPMI-HEPES supplemented with 15% pooled human serum or 15% serum from a specified vaccinated individual. Cultures were established in Microtest II tissue culture plates (Falcon Plastics, Los Angeles, Calif.). Each culture contained 100 μl of a lymphocyte suspension and 100 μl of antigen-containing RPMI-HEPES. The final lymphocyte density was 1.5×10^6/ml of culture. If not otherwise stated, the final density of *F. tularensis* LVS membranes was 10 μg of protein per ml, and the concentration of purified protein derivative 20 μg/ml of culture. Each microplate was closed with a film (Falcon Plastics) and incubated at 37°C for 6 days. The cultures were then pulsed with [^{14}C]thymidine and harvested by the technique described by Hartzman et al. (9) with minor modifications (17). The incorporation of [^{14}C]thymidine has been found to be optimal after about 6 days of incubation with antigen from *F. tularensis* (17).

Agglutination tests. The agglutination test (8) was performed as previously described (17). The antibody titer was expressed as the reciprocal of the highest serum dilution giving agglutination with a clear supernatant.

RESULTS

Lymphocyte response to various doses of *F. tularensis* LVS membranes. Lymphocytes from vaccinated individuals were incubated in the presence of various doses of F. tularensis LVS membranes, and the incorporation of [^{14}C]thymidine into DNA of the lymphocytes was measured. An optimal incorporation was induced by about 10 μg of protein per ml of culture (Fig. 1). This dose was used in the study.

Time of lymphocyte response of *F. tularensis* LVS membranes during the course of tularemia. Lymphocytes were obtained repeatedly from six patients during the first 3 weeks after the onset of fever. They were incubated in the presence and absence of *F. tularensis* LVS membranes, and the incorporation of [^{14}C]thymidine was measured. Lymphocytes obtained 2 weeks or more after onset responded with increased incorporation, whereas lymphocytes obtained earlier after onset did not respond (Fig. 2).

Agglutinating serum antibodies appeared during the end of week 2 after the onset of fever (Fig. 2). Generally, the appearance of antibodies coincided with the appearance of lymphocyte reactivity, although there were discrepancies in individual cases. One patient (Fig. 2) showed no

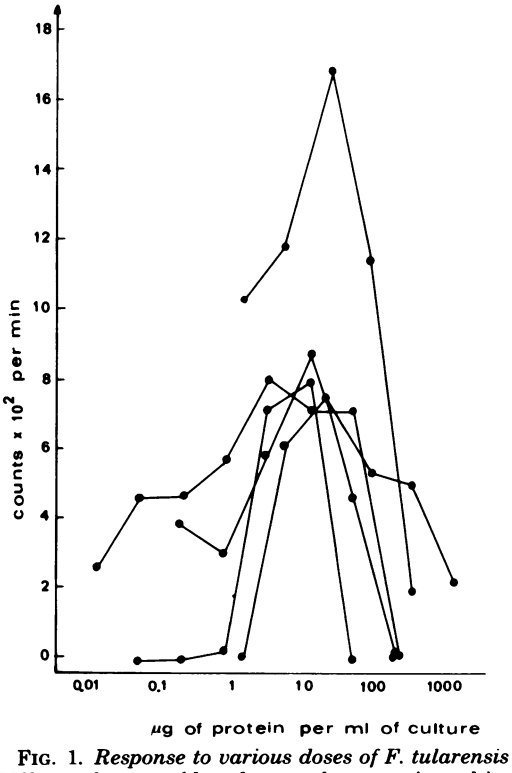

FIG. 1. *Response to various doses of F. tularensis LVS membranes of lymphocytes from vaccinated individuals. Lymphocytes were incubated for 6 days, and the [^{14}C]thymidine incorporation into DNA was estimated. Mean counts per minute of four or five cultures are indicated and the mean counts of cultures without antigen have been subtracted.*

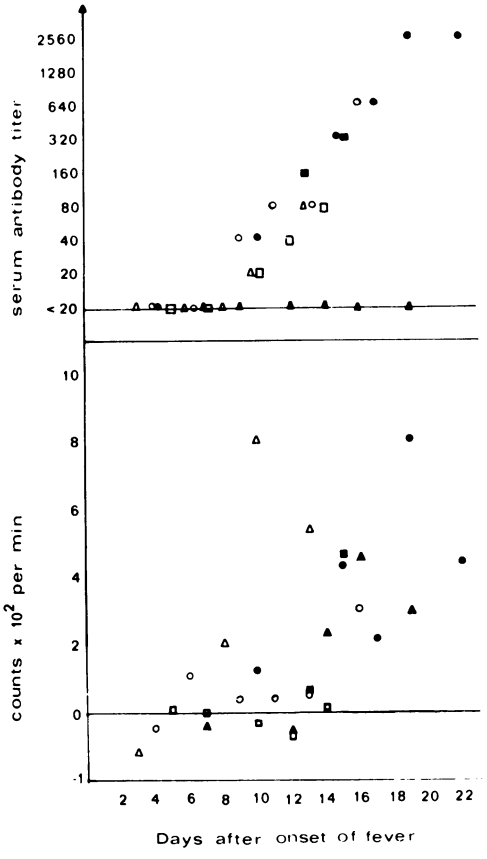

FIG. 2. *Development of lymphocyte reactivity and agglutinating serum antibodies after the onset of tularemia. Lymphocytes were incubated for 6 days in the presence of F. tularensis LVS membranes, and the [^{14}C]thymidine incorporation into DNA was estimated. Mean counts per minute of five cultures are indicated after the subtraction of the mean counts of cultures without antigen. Each kind of symbol represents one patient.*

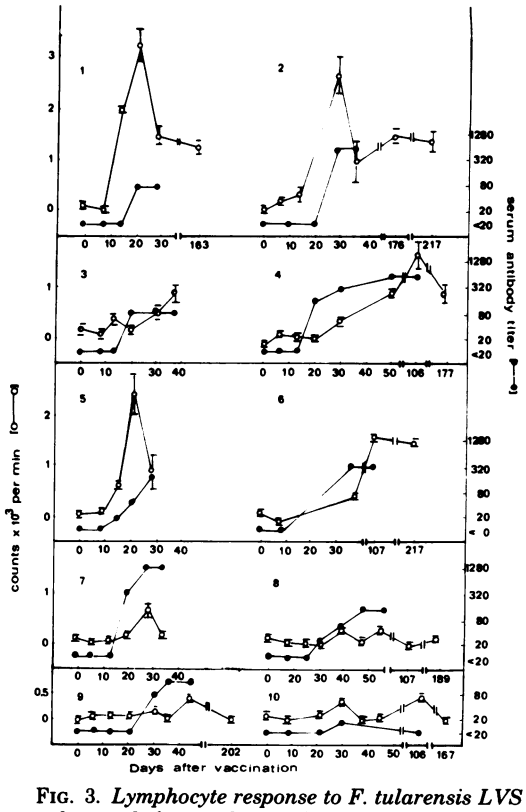

FIG. 3. *Lymphocyte response to F. tularensis LVS membranes before and at various intervals after tularemia vaccination. The incorporation of [^{14}C]thymidine into DNA was estimated, and the means ± standard error of five cultures are indicated* (○), *as well as the titers of agglutinating serum antibodies* (●). *Individuals were numbered from 1 to 10.*

serum agglutinins even on day 19, whereas his lymphocytes responded to the *F. tularensis* LVS membranes. On day 50, however, he had an agglutinin titer of 40.

Time of the lymphocyte response to *F. tularensis* LVS membranes after tularemia vaccination. Lymphocytes obtained before and at various intervals after tularemia vaccination were incubated in the presence and absence of *F. tularensis* LVS membranes, and the incorporation of [^{14}C]thymidine into the DNA of the lymphocytes was estimated. In lymphocytes obtained before vaccination, the membrane-induced incorporation was generally low (Fig. 3). After vaccination, the membranes induced a high incorporation into lymphocytes from 6 of ten individuals (numbers 1 to 6, Fig. 3). The interval between vaccination and the first sample yielding increased [^{14}C]thymidine incorporation varied among the individuals. In lymphocytes from two of them, it occurred after about 2 weeks (numbers 1 and 5), and in the others it occurred after about a month. The incorporation was then consistently high in consecutive samples from these individuals. Lymphocytes from four of them were tested as long as half a year after vaccination and found still to respond well to the membranes (Fig. 3). Lymphocytes of the remaining individuals (numbers 7 to 10) incorporated more [^{14}C]thymidine in the presence than in the absence of membranes on some occasions after vaccination. However, the incorporation was not as high as that of lymphocytes from the other individuals, and it was not consistent in cultures from consecutive samples. Thus, there was a marked variation in the lymphocyte response to the *F. tularensis* LVS mem-

branes among vaccinated individuals, but the ability or inability to respond was reproducible within individuals.

Agglutinating antibodies in serum to *F. tularensis* appeared 2 to 4 weeks after vaccination. Titers in serum from all individuals except one (number 10, Fig. 3) rose to 40 or more. In two of the individuals whose lymphocytes responded well, there was a discrepancy in time between the appearance of serum agglutinins and that of membrane-induced lymphocyte stimulation. In numbers 4 and 1, serum agglutinins and lymphocyte reactivity, respectively, appeared first.

The reasons for the wide variability between individuals in the membrane-induced lymphocyte stimulation after vaccination is unknown. One possibility could be that inhibitory antibodies or other immunoregulatory substances were induced to varying extents in serum by vaccination. Therefore, well-responding lymphocytes (number 1, Fig. 3) were incubated in culture medium containing serum taken before and at various intervals after vaccination of an individual whose lymphocytes responded poorly (number 10, Fig. 3). There was no difference in incorporation of [^{14}C]thymidine between cultures containing serum taken before and at various intervals after vaccination (Fig. 4). Neither was there any difference in incorporation, whether the serum of the culture was from this individual (Fig. 4a) or from an individual (number 5, Fig. 3) whose lymphocytes had been found to respond well to the membranes (Fig. 4b). Similar results were obtained in two additional experiments in which other combinations of sera and lymphocytes were used (data not shown). Thus, no effect of serum was found that might explain the individual variability in the lymphocyte response to *F. tularensis* LVS membranes after vaccination.

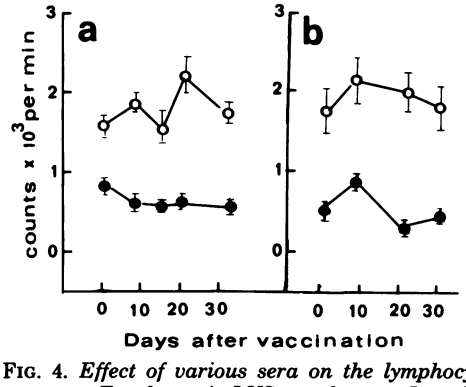

FIG. 4. *Effect of various sera on the lymphocyte response to F. tularensis LVS membranes. Lymphocytes were incubated in the presence of serum obtained at various time intervals after vaccination of one individual (number 10, Fig. 3), whose lymphocytes responded poorly (a) to the membranes and from another individual (number 5, Fig. 3), whose lymphocytes responded well (b). Membranes were present (○) or absent (●). After 6 days of incubation, the incorporation of [^{14}C]thymidine into DNA was estimated, and means ± standard error of five cultures are indicated.*

Lymphocyte response to *F. tularensis* LVS membranes of individuals vaccinated 1 month, 1 year, or 2 years before testing. Lymphocytes from individuals vaccinated 1 month or 1 year before testing responded to *F. tularensis* LVS membranes with similar magnitudes of [^{14}C]thymidine incorporation (Fig. 5). The thymidine incorporation induced into lymphocytes from individuals vaccinated 2 years before testing was, however, significantly ($P < 0.02$) lower than that induced into lymphocytes of individuals vaccinated 1 year previously. There was no difference in purified protein derivative-induced incorporation among the three groups of individuals (Fig. 5).

Also, the agglutinin titers were lower in samples from individuals vaccinated 2 years before testing than in samples from those who had been vaccinated 1 month or 1 year before testing (Fig. 6). The correlation between the two parameters was poor. Thus, the groups vaccinated 1 month, 1 year, or 2 years before the investigation showed correlation coefficients of 0.6, 0.2, and −0.1, respectively. It may be remarked that two of the individuals who lacked demonstrable serum antibodies 2 years after vaccination reacted strongly in the lymphocyte stimulation test (Fig. 6).

DISCUSSION

It was previously reported that lymphocytes from many tularemia-vaccinated individuals responded in vitro to heat-killed bacteria of the vaccine strain, whereas lymphocytes from unvaccinated individuals did not respond (17). The immunospecific nature of the reaction is further established by the present results, since lymphocytes from the same individuals were studied before as well as after vaccination and found to respond to membranes of *F. tularensis* LVS after vaccination only. Furthermore, patients with clinically and serologically verified tularemia developed a lymphocyte reactivity to the membranes during illness.

The magnitude of the lymphocyte response varied widely among the vaccinated individuals. Some responded well and consistently when samples were obtained repeatedly during several months. Lymphocytes from other individuals responded poorly in repeated cultures. The rea-

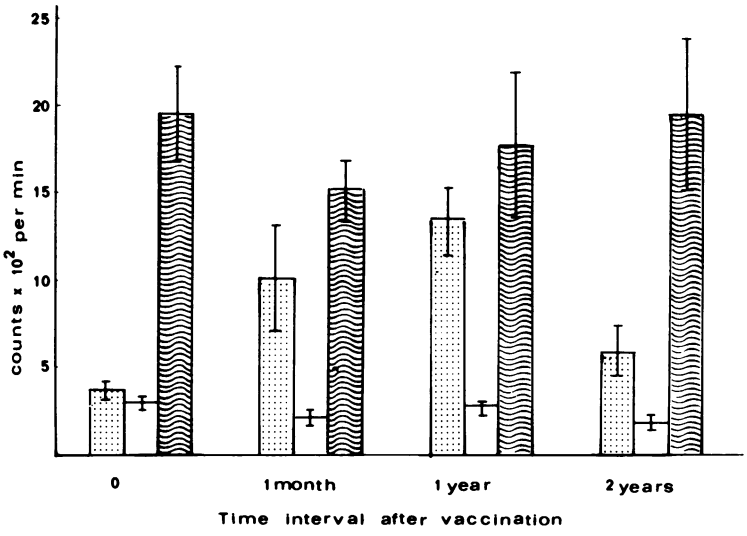

FIG. 5. *Response to F. tularensis LVS membranes of lymphocytes from individuals vaccinated on various occasions before testing. One group of individuals (n = 10) was tested on the day of vaccination as well as 1 month later. Another group (n = 14) had been vaccinated 1 year before testing, and a third group (n = 18) was vaccinated two years before testing. From each individual, five cultures were established with F. tularensis LVS membranes as antigen (dotted bars), five cultures without stimulating agent (open bars), and five cultures with purified protein derivative (lined bars). After 6 days of incubation, the incorporation of [^{14}C]thymidine into DNA was estimated. Counts per minute × 10^{-2} ± the standard error of the mean are indicated.*

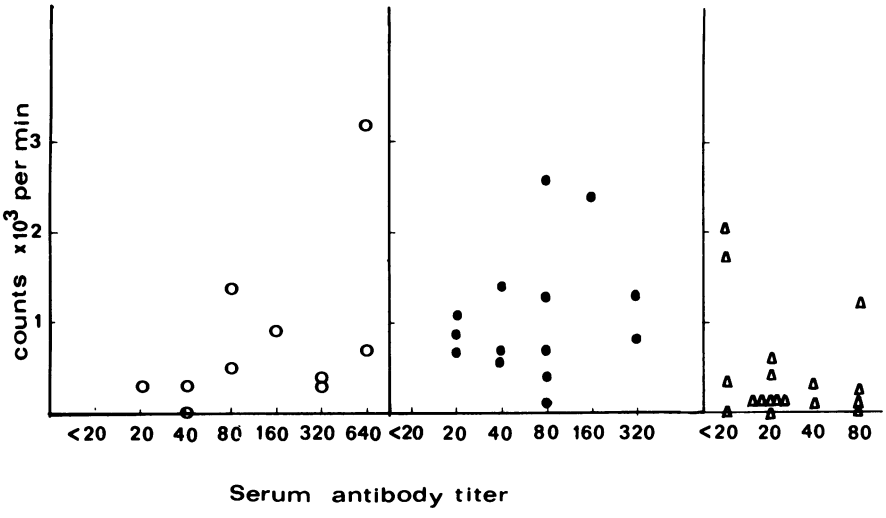

FIG. 6. *Relationship between the magnitudes of lymphocyte response to F. tularensis LVS and titers of agglutinating serum antibodies. Three groups of individuals were investigated. They had been vaccinated 1 month (○), 1 year (●), and 2 years (△) before the investigation. The lymphocyte response was estimated by incorporation of [^{14}C]thymidine into DNA. The mean counts per minute of five cultures are indicated after subtraction of the mean counts of cultures without antigen.*

son for the difference in lymphocyte reactivity among individuals is unknown. It might be due to an individually variable activity of immunoregulatory cells or humoral factors. Suppressive effects of human lymphocyte subpopulations and adherent mononuclear leucocytes have been reported (3, 11). One approach may be to study the relative quantity or activity of suppressor and helper cells in the blood of individuals who respond well and poorly to *F. tularensis* LVS after tularemia vaccination.

Humoral factors may affect the lymphocyte

response to specific antigens (cf. reference 15). Immune complexes of IgM antibodies and rubella antigen have been found to inhibit the response of rabbit lymphocytes to rubella antigens (12). A similar explanation for the poor lymphocyte reactivity of some of the present individuals seems improbable. Thus, serum obtained at various intervals after vaccination of individuals, whose lymphocytes responded poorly, did not affect the response to *F. tularensis* LVS membranes of highly reactive lymphocytes.

The interval between vaccination and the first sample yielding a lymphocyte response to the membrane preparation was 2 weeks in two cases and 1 month in four cases. After the onset of tularemia, there was a period of about 2 weeks before the lymphocyte response appeared in vitro. These results are in contrast to those of a study on vaccination with killed bacteria of *Brucella abortus*, when lymphocytes were found to respond in vitro as early as 3 days after vaccination (2). After BCG vaccination, on the other hand, a lymphocyte response has been demonstrated at day 13 (16). The long interval between the appearance of a lymphocyte response after tularemia vaccination may be due to a suboptimal sensitivity of the assay or to a long duration of the immune process in vivo. Delayed hypersensitivity seems not to appear earlier than 2 weeks after vaccination (5) which indicates that in humans the development of immunity against *F. tularensis* is a relatively slow process. It remains to be established whether a specific lymphocyte response to antigens other than the present may appear earlier.

The lymphocyte stimulation test may be used as a supplement in tularemia diagnostics. It has been found to estimate T lymphocyte reactivity mainly, and its nature is therefore different from that of serological assays. No correlation was found in the present and in a previous study (17) between the magnitude of lymphocyte response and the titer of agglutinating serum antibodies after tularemia vaccination. Two of four vaccinated individuals who lacked demonstrable serum agglutinins 2 years after vaccination responded well in the lymphocyte stimulation test. One of the patients who had a clinically verified tularemia lacked demonstrable serum agglutinins 3 weeks after the onset of disease but responded in the lymphocyte stimulation test.

Cell-mediated immunity is probably a better correlate of host resistance against *F. tularensis* than is the presence of humoral antibody (1, 7, 10, 15, 19). Since the present lymphocyte stimulation test has been found to estimate T lymphocyte reactivity mainly (18), it is reasonable to assume that individuals who respond vigorously have a higher degree of protection than those who respond poorly. This assumption might be tested by investigating vaccinated individuals of high-risk groups, such as workers in risk laboratories. It is well known that the present vaccination procedure does not induce an obligate resistance in the individual against tularemia. Protection is good against the respiratory form but not against the ulceroglandular form of the disease (6). The lymphocyte stimulation test may become one tool in evaluating the effect of vaccination in individual subjects.

According to the present results, cell-mediated immune reactivity may often be demonstrated within a month of vaccination and declines significantly within 2 years of vaccination. Revaccination of risk groups may therefore be indicated only a few years after the first vaccination. However, more efforts should be put toward the standardization of preparations for the lymphocyte stimulation test to get more data before a recommendation of a vaccination schedule can be given.

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Defense Research Institute.

LITERATURE CITED

1. **Allen, W. P.** 1962. Immunity against tularemia: passive protection of mice by transfer of immune tissues. J. Exp. Med. **115**:411–420.
2. **Andersen, V., M. Søborg, and S. F. Sørensen.** 1971. Antigen-induced lymphocyte transformation *in vitro* during primary immunization in man. Acta Pathol. Microbiol. Scand. **79B**:489–494.
3. **Bona, C., and L. Chedid.** 1976. Stimulation of lymphocytes by purified protein derivative: Suppression by cells from humans vaccinated with Bacille Calmette-Guérin. J. Infect. Dis. **133**:465–468.
4. **Böyum, A.** 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation. Scand. J. Clin. Lab. Invest. **21**(suppl):27–50.
5. **Buchanan, T. M., G. F. Brooks, and P. S. Brachman.** 1971. The tularemia skin test. Three hundred twenty-five skin tests in 210 persons: serologic correlation and review of the literature. Ann. Intern. Med. **74**:336–343.
6. **Burke, D. S.** 1977. Immunization against tularemia: analysis of the effectiveness of live *Francisella tularensis* vaccine in prevention of laboratory-acquired tularemia. J. Infect. Dis. **135**:55–60.
7. **Claflin, J. L., and C. L. Larson.** 1972. Infection-immunity in tularemia: specificity of cellular immunity. Infect. Immun. **5**:311–318.
8. **Francis, E., and A. C. Evans.** 1926. Agglutination, cross agglutination and agglutinin absorption in tularemia. U.S. Public Health Dept. **41**:1273–1295.
9. **Hartzman, R. J., F. H. Bach, G. B. Thurman, and K. W. Sell.** 1972. Precipitation of radioactivity labeled samples: a semi-automatic multiple-sample processor. Cell. Immunol. **4**:182–186.
10. **Kostiala, A. A., D. D. McGregor, and P. S. Logie.** 1975. Tularemia in the rat. I. The cellular basis of host resistance to infection. Immunology **28**:855–869.
11. **Kurnick, J. T., C. Bell, and H. M. Grey.** 1976. PHA-induced activation of suppressor cells in normal human

peripheral blood lymphocytes. Scand. J. Immunol. **5:** 771–778.

12. **Lee, J. C., and M. M. Sigel.** 1974. A differential effect of IgM and IgG antibodies on the blastogenic response of lymphocytes to rubella virus. Cell. Immunol. **13:**22–31.
13. **Ling, N. R., and J. E. Kay.** 1975. Immuno-specific activation of lymphocytes of pre-immunized donors, p. 179–200. *in* Lymphocyte stimulation. North-Holland Publishing Co.
14. **Nelson, D. S., and R. A. Gatti.** 1976. Humoral factors influencing lymphocyte transformation. Prog. Allergy **21:**261–341.
15. **Nutter, J. E., and Q. N. Myrvik.** 1966. In vitro interactions between rabbit alveolar macrophages and *Pasteurella tularensis*. J. Bacteriol. **92:**645–651.
16. **Sarkany, I., and H. Hales.** 1968. Lymphocyte transformation following B.C.G. vaccination. Br. J. Dermatol. **80:**29–34.
17. **Tärnvik, A., and S. Löfgren.** 1975. Stimulation of human lymphocytes by a vaccine strain of *Francisella tularensis*. Infect. Immun. **12:**951–957.
18. **Tärnvik, A., and S. E. Holm.** 1978. Stimulation of subpopulations of human lymphocytes by a vaccine strain of *Francisella tularensis*. Infect. Immun. **20:**698–704.
19. **Thorpe, B. D., and S. Marcus.** 1965. Phagocytosis and intracellular fate of *Pasteurella tularensis*. III. *In vivo* studies with passively transferred cells and sera. J. Immunol. **94:**578–585.
20. **Wolf-Watz, H., S. Normark, and G. D. Bloom.** 1973. Rapid method for isolation of large quantities of outer membranes from *Escherichia coli* K-12 and its application to the study of envelope mutants. J. Bacteriol. **115:**1191–1197.
21. **Wolf-Watz, H., T. Elmros, S. Normark, and G. D. Bloom.** 1975. Cell envelope of *Neisseria gonorrhoeae*: Outer membrane and peptidoglycan composition of penicillin-sensitive and -resistant strains. Infect. Immun. **6:**1332–1341.