JOURNAL OF CLINICAL MICROBIOLOGY, June 1987, p. 1074–1078
0095-1137/87/061074-05$02.00/0

Vol. 25, No. 6

Interleukin 2 and Gamma Interferon Production, Interleukin 2 Receptor Expression, and DNA Synthesis Induced by Tularemia Antigen In Vitro after Natural Infection or Vaccination

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Received 17 December 1986/Accepted 9 March 1987

The T-cell response induced by *Francisella tularensis* antigen in sensitized subjects was characterized in vitro by measuring DNA synthesis in whole-blood and mononuclear cell cultures, interleukin 2 (IL-2) and gamma interferon (IFN-γ) production, and IL-2 receptor expression. Correlations between these variables were estimated. The strengths of the responses were compared in 21 subjects naturally infected 2 years ago, 6 subjects vaccinated 5 to 6 years ago, and 13 control subjects with no history of infection or vaccination. Subjects with a history of natural infection synthesized more DNA in both whole-blood and mononuclear cell cultures, secreted more IL-2 and IFN-γ, and expressed more IL-2 receptors than control subjects did. All these responses differed highly significantly ($P < 0.001$) from those of the control subjects. The vaccinees exhibited somewhat lower responses than the naturally immunized subjects did, but the vaccinees could be distinguished from the control subjects by their DNA synthesis, receptor expression, and IFN-γ production ($P < 0.01$ to 0.001). The vaccinees showed a lower response, in terms of DNA synthesis and IL-2 secretion ($P < 0.05$), than the infected group did but responded in a manner similar to that of this group, with respect to receptor positivity and IFN-γ secretion ($P > 0.10$). The correlations between all the T-cell functions were good, with highly significant correlations ($P < 0.001$) between whole-blood DNA synthesis and IL-2 and IFN-γ secretion and between the two lymphokines ($P < 0.001$). The results not only increase our knowledge of the T-cell response to tularemia antigen but also give an alternative approach to DNA synthesis measurement for the quantitation of T-cell responses. The results for the low-responding sensitized subjects seem to indicate that the parameters were comparable in sensitivity.

Francisella tularensis is a small gram-negative bacillus which causes sporadic or epidemic infections in humans. Diagnosis is usually confirmed by an antibody rise in the agglutination test (21), but it is the cell-mediated immune response that is mainly responsible for resistance to infection (1, 4, 16, 26). The cellular response can be measured in vivo by the delayed-type skin test (8) or in vitro by the DNA synthesis measurement by using either mononuclear cell or whole-blood cultures (14, 15, 23, 25). Cell-mediated immunity seems to be long-lasting, since the DNA synthesis response has been shown to be positive 11 years after infection (14) and 9 years after vaccination (24).

Mitogens or specific antigens initiate T-cell responses by inducing both interleukin 2 (IL-2) release from activated cells and the expression of specific receptors on cells responding to IL-2. Activated T-cells also secrete other lymphokines, including gamma interferon (IFN-γ) (20).

We have reported previously on the presence of IL-2 in supernatants from cultures of whole blood containing tularemia antigen from seven subjects vaccinated against the bacteria (12), and we now go on to characterize the in vitro T-cell response induced by *F. tularensis* antigen in naturally infected and vaccinated subjects by measuring IL-2 and IFN-γ production, IL-2 receptor expression, and DNA synthesis in cultures of isolated mononuclear cells and whole blood.

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MATERIALS AND METHODS

Subjects. A total of 21 subjects with a history of serologically confirmed tularemia infection in 1983 (age, 45 ± 13 years) were tested, together with 6 subjects vaccinated with live attenuated bacterial vaccine (U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md.) in 1979 and 1980 (age, 40 ± 8 years) and 13 control subjects with no history of infection or vaccination (age, 37 ± 9 years). The various tests were performed in parallel from heparinized blood samples, with samples from both the sensitized and control groups included in each test series.

Reagents. The tularemia antigen consisted of Formalin-killed whole bacteria (the vaccine strain). Other stimulants were phytohemagglutinin (PHA) (Difco Laboratories, Detroit, Mich.), concanavalin A (Pharmacia Fine Chemicals AB, Uppsala, Sweden), and purified protein derivative of tuberculin (PPD) (Statens Seruminstitut, Copenhagen, Denmark). Lymphocult-T-LF (Biotest Diagnostics, Frankfurt, Federal Republic of Germany) was used as a standard supernatant containing IL-2. RPMI 1640 containing 10% inactivated human AB serum was used as the culture medium. Phycoerythrin-conjugated anti-IL-2 receptor monoclonal antibody was purchased from Becton Dickinson and Co. (Mount View, Calif.).

Measurement of DNA synthesis. Peripheral blood mononuclear cells (PBMC) were separated by centrifugation on

Lymphoprep gradients. A 50-μl quantity of whole blood diluted 1:4 in RPMI 1640 or a PBMC suspension (1×10^6 cells per ml of medium) was cultured with 50 μl of tularemia antigen (final concentration, 150 μg/ml) and medium to a total volume of 200 μl in microtiter plate wells for 7 days in a humidified 5% CO_2 atmosphere. [^{3}H]thymidine (0.4 μCi/well; Amersham International plc, Amersham, United Kingdom) was present for the last 24 h of the incubation time. Median values (counts per minute) for triplicate wells were used for the calculations.

Preparation of IL-2 and IFN-γ supernatants. PBMC at a final concentration of 1×10^6 cells per ml of medium were cultured with tularemia antigen in a total volume of 2 ml (for IL-2) or 600 μl (for IFN-γ) in tissue culture tubes. The incubation times were 24, 48, and 72 h for the IL-2 supernatants and 24, 48, 72, 96, and 120 h for the IFN-γ supernatants. The supernatants were centrifuged and frozen at −20°C until tested.

IL-2 assay. Concanavalin A-stimulated lymphoblasts were used as test cells in the assay. PBMC from a healthy person were prepared and stimulated with concanavalin A (20 μg/ml) for 5 days, after which the cells were washed and the number of living cells was counted. These cells (10^4) in 100 μl of medium were cultured with 100 μl of various dilutions (1:1, 1:2, and 1:4) of the supernatants to be tested. An incubation of 48 h was used, with [^{3}H]thymidine present for the last 24 h. The control Lymphocult-T-LF supernatant containing IL-2 was included to show the response of the test cells (13). Control wells included cultures of test cells with both medium and amounts of tularemia antigen comparable to those in the supernatant dilutions. The samples were tested in triplicate. The values (counts per minute) for 1:1 dilutions are shown in the results, since these fell on the linear part of the dilution curve. [^{3}H]thymidine incorporation into the control cultures with tularemia antigen did not differ from that observed in the test cells in medium (median, 520 cpm). These counts were not subtracted from the values expressed in the results.

IFN-γ assay. IFN-γ was measured with an enzyme-linked immunosorbent assay using two mouse monoclonal antibodies, designated 1-D1K and 7-B6-1, produced in our own laboratories after immunization of BALB mice with human recombinant IFN-γ (produced by Genentech, Inc., San Francisco, Calif., and kindly provided by Boehringer Ingelheim, Vienna, Austria). Microtiter plates were coated overnight with 100 μl of monoclonal antibody 1-D1K diluted to 2 μg/ml with phosphate-buffered saline (PBS), pH 7.4, at +4°C, followed by incubation with 1% bovine serum albumin in PBS for 1 h at room temperature. Aliquots (100 μl) of the samples and standards diluted in 1% bovine serum albumin–PBS were then incubated for 4 h at room temperature. The second monoclonal antibody, 7-B6-1, conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) was added for the detection of bound human IFN-γ. The plates were kept at room temperature overnight and developed with *p*-nitrophenylphosphate substrate (Sigma), and the absorbances were read at 405 nm in a Multiskan automated spectrophotometer (Flow, Irvine, Scotland). Washes with 0.9% NaCl–0.05% Tween 20 were performed between the incubation steps. The standard used was the international human IFN-γ standard Gg 23-901-530, kindly supplied by the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

IL-2 receptor induction and measurement. Mononuclear cells (4×10^5) were cultured in tubes in a total volume of 400 μl (stimulant included) to express the IL-2 receptors. The cells from 20 subjects (including infected, vaccinated, and control individuals) were stimulated with each of the three stimulants (tularemia antigen, final concentration as above; PHA, final concentration, 10 μg/ml; PPD, final concentration, 10 μg/ml). The cells from the remaining 20 subjects were stimulated only with tularemia antigen. To measure IL-2 receptor expression, the cells were stained with phycoerythrin-conjugated monoclonal IL-2 receptor antibody (Becton Dickinson) before incubation and after 2, 4, and 7 days of stimulation. After centrifugation, the supernatants were discarded, and the cells were incubated with 20 μl of antibody for 30 min at 4°C, washed once with PBS containing fetal bovine serum and azide, and placed on microscope slides. The percentages of positive cells were determined by counting 200 living mononuclear cells per slide with alternating phase-contrast and fluorescence illumination.

Statistical methods. The Mann-Whitney U test was used to calculate the differences between the three groups of subjects. Correlations were determined and tested using Spearman's rank correlation coefficient.

RESULTS

Median values for the variables. The subjects with a history of natural infection differed highly significantly from the control subjects ($P < 0.001$) with respect to tularemia antigen-induced DNA synthesis in both whole-blood and mononuclear cell cultures, to the production of IL-2 and IFN-γ in these cultures, and to IL-2 receptor expression in 4- and 7-day cultures (Table 1). IL-2 secretion in subjects with a history of natural infection peaked on day 2. The mononuclear cells from the vaccinated subjects synthesized more DNA in whole-blood and separated-cell cultures ($P < 0.001$), expressed more IL-2 receptors in 4- and 7-day cultures ($P < 0.01$), and produced more IFN-γ ($P < 0.001$) than did those from the control subjects; they also showed a tendency to secrete more IL-2, but the difference was not statistically significant ($P < 0.10$). The response of vaccinees was lower than that of the postinfection group in terms of DNA synthesis (whole-blood synthesis, $P < 0.05$; mononuclear cell synthesis, $P < 0.01$) and IL-2 production ($P < 0.05$), but the two groups were comparable in receptor expression in 4- and 7-day cultures ($P > 0.10$) and in IFN-γ secretion ($P > 0.10$).

There were four subjects with a disease or immunization history whose whole-blood blastogenesis was low (less than the mean plus 2 standard deviations of the response in the control subjects). One of these produced IL-2 and another produced IFN-γ, but none had receptor expression above the 95% confidence interval for the control subjects (data not shown).

Kinetics of IFN-γ production. Tularemia antigen induced IFN-γ secretion in the naturally infected subjects, while the control subjects had no production on any day, as shown in Fig. 1. The difference between the naturally infected and control subjects was significant after only 24 h ($P < 0.01$), and maximum secretion was recorded on day 4. The vaccinated subjects also produced more IFN-γ in 2- to 5-day cultures ($P < 0.01$ or 0.001), and the IFN-γ production kinetics and median quantities resembled those in naturally infected subjects (data not shown).

Induction of IL-2 receptors by tularemia antigen, PPD, and PHA. The kinetics and strength of the responses induced by the tularemia antigen were compared with those induced by another antigen, PPD, and by the polyclonal stimulant PHA

TABLE 1. Various T-cell functions induced by *F. tularensis* antigen in vitro[a]

Subjects	DNA synthesis (cpm)		IL-2 (cpm) at day 2	IFN-γ (U/ml) at day 5	% IL-2 receptors at:	
	Whole blood	Lymphocyte			Day 4	Day 7
After natural infection						
Median	19,326	38,324	1,402	100	7	18
Range	97–42,578	2,205–96,997	189–6,549	0–616	0.5–23	5–47.6
n	21	21	21	19	21	21
Vaccinated						
Median	1,075	14,265	421	80	7.4	14
Range	348–10,779	4,526–29,629	220–1,973	12–233	3.5–15	2–34
n	6	6	6	6	6	6
Control						
Median	219	1,471	241	0	2.5	4
Range	31–445	241–8,021	119–1,079	0–28	0.5–10	0.5–7.5
n	13	13	13	12	13	13

[a] According to the Mann-Whitney U test, between subjects with a history of natural infection and control subjects the *P* values were <0.001 for all variables. For the quantities of DNA synthesis in whole-blood and in lymphocyte cultures, of IL-2 and IFN-γ, and of IL-2 receptors in 4- and 7-day cultures, the *P* values, respectively, were <0.001, <0.001, <0.10, <0.001, <0.01, and <0.01 for vaccinated and control subjects and <0.05, <0.01, <0.05, >0.10, >0.10, and >0.10 for subjects with a history of natural infection and vaccinated subjects.

(Fig. 2). Tularemia- and PPD-induced receptors (18 versus 25%) peaked on day 7, while the highest percentage of receptor-positive cells resulting from PHA stimulation (61%) was achieved on day 4.

Correlations between variables. DNA synthesis in whole blood was significantly correlated with that in the PBMC cultures ($r = 0.709$, $P < 0.01$), but whole-blood DNA synthesis correlated more closely with lymphokines than did lymphocyte DNA synthesis (coefficient level, 0.696 to 0.925 versus 0.266 to 0.661). The correlations between whole-blood blastogenesis and IL-2 or IFN-γ were significant ($P < 0.01$) or highly significant ($P < 0.001$). Whole-blood synthesis correlated with the 4-day receptor percentage ($r = 0.598$, $P < 0.01$) but not with the 7-day percentage ($r = 0.326$, $P > 0.10$). The quantities of IL-2 (1, 2, or 3 days) and IFN-γ produced (3, 4, or 5 days) showed highly significant correlations ($r = 0.755$ to 0.852, $P < 0.001$). A diagram relating IL-2 after 2 days and IFN-γ after 4 days is given in Fig. 3 ($r = 0.851$, $P < 0.001$).

DISCUSSION

A cell-mediated immune response to tularemia antigen could be demonstrated in subjects with a history of natural infection in the form of IFN-γ and IL-2 production and IL-2 receptor expression in PBMC cultures and in a standard blast transformation test measuring DNA synthesis in either mononuclear cell or whole-blood cultures. A lower level of responsiveness was found in vaccinees than in the subjects with natural infection, as far as IL-2 secretion and the results of the DNA synthesis test were concerned. This could reflect the difference in the time elapsing since immunization, which

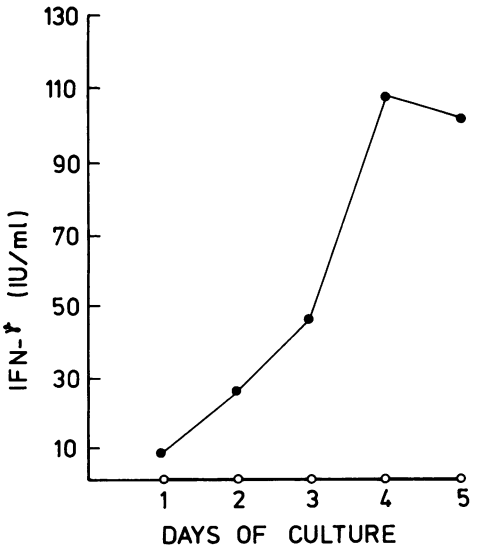

FIG. 1. Kinetics of tularemia antigen-induced IFN-γ production. Median values in subjects after natural infection (●) and in control subjects (○) are plotted.

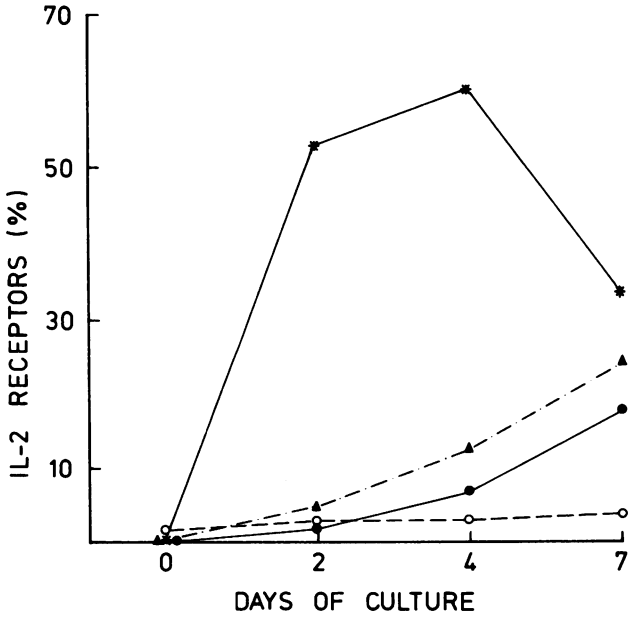

FIG. 2. Kinetics of IL-2 receptor expression. Median values in cultures induced with PHA (*), PPD (▲), and tularemia antigen (●, subjects after natural infection; ○, control subjects) are plotted.

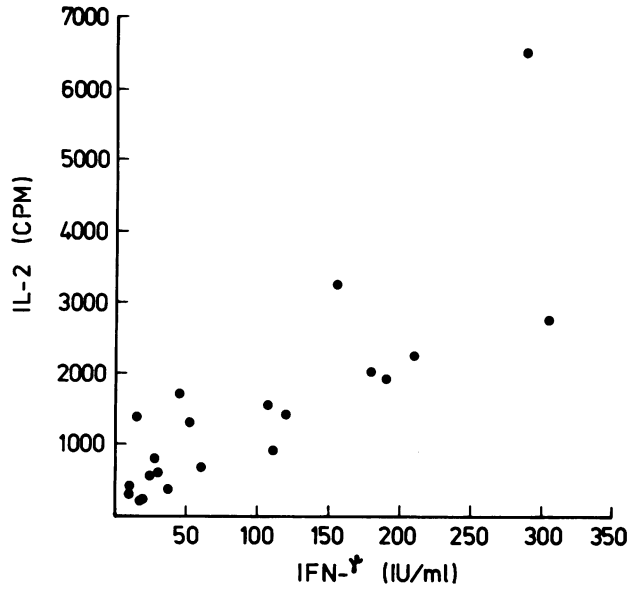

FIG. 3. Correlation between the quantities of IL-2 in 2-day cultures and IFN-γ in 4-day cultures. $r = 0.85$, $P < 0.001$.

took place 3 or 4 years earlier in the vaccinees than in the subjects with natural infection, but a more probable explanation is that a stronger response is obtained by natural infection. This is supported by the results of Koskela and Herva (15) and Tärnvik et al. (24), which show that the DNA synthesis response remains at the same level years after infection or vaccination, respectively. On the other hand, the vaccination and postinfection groups did not differ in IFN-γ production and IL-2 receptor expression.

The results again confirm that whole-blood culture is a more specific method than PBMC culture, for the counts from whole-blood cultures of control subjects were low and did not exceed those recorded without antigen, as happened in the PBMC cultures. Sandström et al. (19) described a purified bacterial protein component which does not induce nonspecific stimulation, and thus it seems that more purified antigens are preferable when using mononuclear cell rather than whole-blood cultures.

The finding that correlations between DNA synthesis and the production of lymphokines were higher when the results obtained using the whole-blood culture method were used underlines the specificity of lymphokine measurements and suggests that the nonspecific proliferation induced by whole bacterial antigen may reflect non-T-cell proliferation.

The occurrence of the peak in IL-2 production in the 2-day cultures is in agreement with results regarding virus-induced IL-2 secretion (2, 11), while the IFN-γ kinetics obtained here are in accordance with results from stimulation induced by cytomegalovirus, allogeneic cells, and malaria antigen (5, 10, 27). The kinetics of IL-2 and IFN-γ also agree with the notion of IFN-γ being induced and regulated by endogenous IL-2 (28). The quantity of IFN-γ was more useful in differentiating the immune groups from the control subjects than that of IL-2, but this may have been brought about by the difference in assay methods.

The IL-2 receptor percentage and kinetics depended on the type of stimulus (polyclonal versus antigen induced). The percentages obtained in the PPD and tularemia cultures show that both antigens induce strong lymphoblast proliferation responses. The reason for using PPD as a stimulant is the Finnish *Mycobacterium bovis* BCG vaccination program, which covers the entire population in infancy and causes positive T-cell responses in vitro in practically all subjects. The appearance of receptors was slow in the antigen cultures, and the immune cells had no more receptor positivity in 2-day cultures with tularemia antigen than did the control cells, despite maximal IL-2 production at that time. The phenotype of receptor-positive cells has not been established, but helper (CD4-positive) cells are probable candidates. Double fluorescence staining should reveal the nature of the proliferating cells.

Close correlations were found here between DNA synthesis, lymphokine production, and IL-2 receptor expression. Immune-specific IFN-γ production has been reported in a number of virus stimulations (2, 3, 5–7, 9, 17, 18, 22) and in allogeneic cell cultures (10), but less work has been done comparing microbial T-cell variables other than those induced by viral antigens. Stimulation of T-cells from acutely infected malaria patients by using crude *Plasmodium falciparum* antigen has been characterized recently (27), but although a correlation was reported between DNA synthesis and IL-2 production, IFN-γ production and DNA synthesis did not correlate. Interestingly, the production of IFN-γ seemed to be a sensitive indicator of antigen-reactive T-cells since it was found in cultures from immune donors.

In conclusion, lymphokine quantification and IL-2 receptor staining seem to be valid alternatives to DNA synthesis measurement and sensitive ways of detecting tularemia antigen-specific T-cell activation in vitro. Discrimination between different aspects of cellular immune reactions in relation to the clinical picture may aid in analyzing the protective and possible harmful components of responses to various pathogens.

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