CD69 Expression on CD4⁺ T Lymphocytes after In Vitro Stimulation with Tuberculin Is an Indicator of Immune Sensitization against Mycobacterium tuberculosis Antigens

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The expression of the CD69 antigen on CD4 T lymphocytes after in vitro stimulation with purified protein derivative (2 tuberculin units) was used to evaluate the tuberculin reactivities of 52 individuals from four experimental groups: Mycobacterium bovis BCG-vaccinated healthy individuals with a negative tuberculin skin test (TST) result (group A), BCG-vaccinated healthy individuals with a positive TST result (group B), patients with active tuberculosis (TB) before treatment (group C), and individuals with clinically inactive TB who had previously completed a prescribed course of chemotherapy (group D). The expression of CD69 on CD4 T lymphocytes was significantly higher in patients with active TB (16.2% ± 7.3%), individuals with clinically inactive TB (10.5% ± 7.4%), and healthy individuals with a positive TST result (15.5% ± 7.2%) than in healthy individuals with a negative TST result (3.8% ± 4.3%) (P < 0.005). We confirmed the correlation between CD69 antigen expression on T lymphocytes after stimulation with tuberculin and the TST induration diameter (Spearman rho = 0.783; P < 0.001), an assay for gamma interferon (the Quantiferon-TB assay; Spearman rho = 0.613; P < 0.001), and the lymphocyte BLAST transformation test (Spearman rho = 0.537; P < 0.001). Our results demonstrate the usefulness of the determination of CD69 on CD4 T lymphocytes after in vitro stimulation with tuberculin as a rapid indicator of immune sensitization against Mycobacterium tuberculosis.

The fast, early, and accurate diagnosis of Mycobacterium tuberculosis infection is a very important element of global health measures for the control of tuberculosis (TB). The identification of individuals with latent M. tuberculosis infection (LTBI) who will benefit from treatment is crucial to the goal of TB elimination (1, 2). Since the immune response to mycobacterial infection is predominantly cellular (11), assessment of whether a patient’s T cells have been exposed to and sensitized by antigens specific to M. tuberculosis provides an approach to diagnosis (4). Delayed-type hypersensitivity skin testing by the tuberculin skin test (TST) with purified protein derivate (PPD) is the standard method of screening for TB and has been a convenient, cost-effective method for evaluation of the lymphocyte effector function (39). The expression of the CD69 antigen has been identified as the earliest activation marker on the surfaces of antigen- or allergen-specific activated lymphocytes in vitro (36). Once CD69 is expressed, it acts as a costimulatory molecule for T-cell activation and proliferation (39). The expression of the early lymphocyte activation marker CD69 after stimulation with tuberculin has been evaluated in healthy individuals with positive and negative TST results and has been shown to correlate well with the TST induration diameter (30). A significantly higher level of expression of CD69 was found in patients with TB 8 months after they started treatment compared to that in healthy controls (26). Furthermore, in patients coinfected with HIV type 1 and M. tuberculosis, the expression of CD69 correlated with the results of TST and IFN-γ production in PPD-stimulated CD4 T lymphocytes (5, 22).

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T lymphocytes. Because little is known about CD69 antigen expression on CD4 T lymphocytes after in vitro tuberculin stimulation in patients with proven active TB before treatment and individuals with clinically inactive TB and a history of a previous episode of TB, we designed a study to evaluate the expression of CD69 on CD4 T lymphocytes using flow cytometry. Patients with active TB before treatment, individuals with clinically inactive TB who had completed a previously prescribed course of chemotherapy, and M. bovis BCG-vaccinated healthy individuals with a positive or a negative TST reaction were included in the study. Furthermore, we compared the expression of CD69 on CD4 T lymphocytes with the microbiological and clinical diagnoses, with the results of the in vitro lymphocyte BLAST transformation test (LT)T, the QFT results, and the induration size by TST.

MATERIALS AND METHODS

Subjects. Fifty-two Slovenian individuals vaccinated with BCG (25 females and 27 males) were evaluated over a 7-month period. In Slovenia BCG vaccination is included in the regular national vaccination program and is obligatory for newborns. Every child is vaccinated on the third day after birth. The participants were divided into the following four groups. Group A consisted of 15 healthy individuals (8 females and 7 males) with a negative TST reaction. Group B consisted of 16 healthy individuals (6 females and 10 males) with a positive TST reaction. All healthy individuals (groups A and B) were volunteers. None of the individuals in these two groups had a history or evidence of M. tuberculosis infection or exposure to M. tuberculosis as the time of inclusion in the study. Group C consisted of six patients (three females and three males) with a clinically active first episode of pulmonary TB for whom diagnostic procedures had been completed. They were included in the study before the initiation with tuberculin was compared to the TST induration diameter. The induction of CD69 expression on CD4 T lymphocytes after stimulation with tuberculin was compared to the TST induration diameter. The interpretive criteria for positive TST results of the Centers for Disease Control and Prevention were followed (2). For groups C and D, the expression of CD69 on CD4+ T lymphocytes after stimulation with tuberculin was compared to the clinical diagnosis. In all groups, the expression of CD69 on CD4+ T lymphocytes after stimulation with tuberculin was compared to the QFT and LT results. The distribution of the data was determined with a skewness coefficient. P values less than 0.05 were considered statistically significant. The SPSS for Windows statistical program (version 10.0) was used for statistical analysis.

RESULTS

Demographic data. The age range of the subjects included in the study was 28 to 73 years, and the mean age was 49.3 years (standard deviation, 13.1 years). The age range and the mean age of subjects and their TB infection status are shown in Table 1, by group.

CD69 surface expression on activated CD4+ T lymphocytes in whole-blood samples stimulated with tuberculin measured by flow cytometry. CD4 T lymphocytes in whole-blood samples from four different groups were activated with tuberculin and expressed different levels of the CD69 activation antigen.

A statistically significantly higher level of expression of the CD69 antigen on CD4+ T lymphocytes was found in the group of patients with active TB (group B), individuals with clinically inactive TB (group D), and healthy individuals with a positive TST reaction (group B) than in the group of healthy individuals with a negative TST reaction (group A) (F = 9.95; P < 0.005). There were no significant differences in the levels of expression of the CD69 activation antigen between groups B, C, and D (Fig. 1, upper left panel). An overview of the in vitro test results is given in Table 1.

Correlation between expression of CD69 on CD4+ T lymphocytes in whole-blood samples stimulated with tuberculin and TST induration diameter in groups of healthy individuals. The expression of CD69 on CD4+ T lymphocytes after in vitro activation with tuberculin in groups of BCG-vaccinated healthy
Correlation between expression of CD69 on CD4+ T lymphocytes in whole-blood samples stimulated with tuberculin and in vitro tests. The correlation between the extent of CD69 expression on CD4+ T lymphocytes after in vitro activation with tuberculin and the LTT results was significant (Spearman rho = 0.537; P < 0.001). The correlation between the extent of CD69 expression on CD4+ T lymphocytes after in vitro activation with tuberculin and the QFT results was significant (Spearman rho = 0.421; P = 0.001). The correlation between the extent of CD69 expression on CD4+ T lymphocytes after stimulation with tuberculin and the TST induration diameter in groups of BCG-vaccinated healthy individuals was statistically significant. This result is consistent with the results of previous study (30) that demonstrated a good correlation between the expression of the early lymphocyte activation antigen CD69 on CD4+ T lymphocytes after stimulation with tuberculin and the TST induration diameter in groups of BCG-vaccinated healthy individuals with positive and negative TST reactions. The TB infection status in BCG-vaccinated healthy individuals with a positive TST reaction is not clear, because there is no reliable method of distinguishing tuberculin reactions caused by vaccination with BCG from those caused by natural mycobacterial infections. BCG-vaccinated people may test positive by the Mantoux skin test, even if they are not latently infected with TB. In comparison, the M. tuberculosis infection status of our study individuals in groups C and D were known. TB was confirmed by clinical findings, radiography, and isolation of M. tuberculosis in culture.

The correlation between the expression of CD69 on CD4+ T lymphocytes after in vitro stimulation with tuberculin and the QFT results was significant. In contrast to the results of the QFT, the highest levels of expression of CD69 were observed in the group of patients with culture-proven active TB before treatment. The mean values for the expression of CD69 in groups of patients with clinically inactive TB and healthy individuals with a positive TST reaction were, however, lower. The highest levels of IFN-γ in our study, as well as in previous

TABLE 1. Clinical characteristics, TB status, expression of CD69 on CD4+ T lymphocytes after stimulation with tuberculin, and results of QFT and LTT with whole-blood samples from individuals in groups A to D

| Characteristic                        | Result for group:       |
|---------------------------------------|-------------------------|
|                                       | A (n = 15)               |
|                                       | B (n = 16)               |
|                                       | C (n = 6)                |
|                                       | D (n = 15)               |
| Age (yr)                              |                         |
| Mean ± SD                             | 54 ± 14.6               |
| Range                                 | 28–69                   |
|                                       | 47.6 ± 12.4             |
|                                       | 30–73                   |
| M. tuberculosis infection status       | TST negative            |
|                                       | TST positive            |
|                                       | Culture-proven TB       |
|                                       | Culture-proven TB       |
| % CD4+ T lymphocytes with CD69 expression |                         |
| Mean                                  | 3.8                     |
| SD                                    | 4.3                     |
| QFT result (%)                        |                         |
| Mean                                  | 10.4                    |
| SD                                    | 12.9                    |
| LTT result (cpm)                      |                         |
| Mean                                  | 575                     |
| SD                                    | 819                     |

DISCUSSION

The expression of the early activation antigen CD69 on CD4+ T lymphocytes after stimulation with tuberculin was measured to answer the question of whether detection of this antigen provides a quicker, easier, and more relevant method for evaluation of sensitization to M. tuberculosis antigens.

We found a high level of expression of the CD69 antigen on CD4+ T lymphocytes in the group of patients with active TB, patients with inactive TB, and healthy subjects with a positive TST reaction. The statistical analysis confirmed that the measurement of CD69 on CD4+ T lymphocytes provides a relevant method for the detection of sensitization to M. tuberculosis. The lymphocytes of healthy subjects with a negative TST reaction expressed statistically significantly lower quantities of the CD69 antigen on CD4+ T lymphocytes after in vitro stimulation with tuberculin. No significant differences in the expression of CD69 on CD4+ T lymphocytes after activation with tuberculin were found between patients with active disease before treatment, patients with clinically inactive TB, and healthy individuals with a positive TST reaction.

According to our results, the level of activation of the CD69 antigen on CD4+ T lymphocytes after stimulation with tuberculin is a direct indicator of immune sensitization against the mycobacterial antigens. The correlation between the expression of CD69 on CD4+ T lymphocytes after activation with tuberculin and the TST induration diameter in groups of BCG-vaccinated healthy individuals was statistically significant. This result is consistent with the results of previous study (30) that demonstrated a good correlation between the expression of the early lymphocyte activation antigen CD69 on CD4+ T lymphocytes after stimulation with tuberculin and the TST induration diameter in groups of BCG-vaccinated healthy individuals with positive and negative TST reactions. The TB infection status in BCG-vaccinated healthy individuals with a positive TST reaction is not clear, because there is no reliable method of distinguishing tuberculin reactions caused by vaccination with BCG from those caused by natural mycobacterial infections. BCG-vaccinated people may test positive by the Mantoux skin test, even if they are not latently infected with TB. In comparison, the M. tuberculosis infection status of our study individuals in groups C and D were known. TB was confirmed by clinical findings, radiography, and isolation of M. tuberculosis in culture.

The correlation between the expression of CD69 on CD4+ T lymphocytes after in vitro stimulation with tuberculin and the QFT results was significant. In contrast to the results of the QFT, the highest levels of expression of CD69 were observed in the group of patients with culture-proven active TB before treatment. The mean values for the expression of CD69 in groups of patients with clinically inactive TB and healthy individuals with a positive TST reaction were, however, lower. The highest levels of IFN-γ in our study, as well as in previous
studies (8, 26, 32, 37, 38), were observed in the group of healthy individuals with a positive TST reaction and patients with clinically inactive TB. The mean levels of IFN-γ secreted by the group of patients with culture-proven active TB before treatment were lower. The exact mechanisms responsible for the differences are not known. Some studies have implied a phenotype shift of Th precursors to the Th2 type in patients with active TB and lower levels of IFN-γ production (14, 28, 34, 35). Stimulation with tuberculin in patients with active TB may induce T lymphocytes to express the early activation antigen but not to express IFN-γ or to proliferate (6, 21). As expected, healthy individuals with a negative TST reaction also produce low levels of this cytokine in response to tuberculin (32).

In vitro tests which evaluate a segment of the complex cell-mediated immunologic events required to produce cutaneous induration in tuberculin-positive individuals have several inherent advantages: they require only a single patient visit, they lack a booster effect, and their interpretation is more objective. In vitro techniques require shorter times (18 to 24 h) of incubation of the patient’s blood with tuberculin and are technically simple to perform. They could also be useful for monitoring the response to treatment (31) and as screening tests for tuberculin reactivity in high-risk groups. By virtue of their ability to quantitate the differential responses to different mycobacterial antigens, they have the potential to discriminate between different mycobacterial infections. Sepkowitz (29) reported that the size of the TST reaction correlated with the risk of development of active TB. Strong responders demonstrated a higher incidence of development of active disease (29). Thus, the ability to measure CD69 expression and to quantify the reaction to tuberculin might help estimate the risk of development of active disease. Their simplicity and rapidity suggest that they might offer advantages over TST.

The cutoff value characteristic for the clinically relevant expression of CD69 on CD4+ T lymphocytes has not yet been determined. Further investigations need to be undertaken to
determine the applicability of the measurement of the CD69 antigen to the determination of tuberculin reactivity and the detection of LTBI. As with TST and QFT, the interpretation and indicated applications of the measurement of the expression of CD69 on CD4+ T lymphocytes would differ among individuals, according to their risk of LTBI and the development of TB. Some studies have demonstrated the usefulness of the CD69 measurement in assessing the T-lymphocyte function in immunodeficiency states (18, 25). Measurement of CD69 expression could therefore be used to determine the tuberculin reactivities of individuals in groups at high risk for M. tuberculosis infection without concern for artificial boosting of the response and false-positive interpretation of the skin test result due to repeated TST testing. When individuals in immunocompromised groups at high risk for TB are tested, lowering of the test cutoff value would be necessary to increase the sensitivity of detection. The testing of substantially larger numbers of individuals with known TB infection status by the assay will be required to determine the most appropriate cutoff between positive and negative reactors.

A significant drawback of both TST and in vitro tests is the nonspecific response to tuberculin due to cross-reactivity between tuberculin and other mycobacterial species. The false positivities of the in vitro tests for BCG-vaccinated individuals could be overcome by using more specific antigens instead of crude protein preparations of tuberculin. Low-molecular-mass secreted antigens ESAT-6 and CFP-10 of substantially larger numbers of individuals with known TB status will be required to address its sensitivity, specificity, and clinical and epidemiological relevance and to determine the most appropriate cutoff between positive and negative reactivities by this assay.

In conclusion, this study demonstrates the potential of in vitro assays for the detection of TB in humans. We have demonstrated the usefulness of the measurement of the early activation antigen CD69 on CD4+ T lymphocytes after in vitro stimulation of whole blood with tuberculin as a marker for tuberculin reactivity and TB. The measurement of CD69 on CD4+ T lymphocytes after in vitro stimulation with low-molecular-mass secreted antigens ESAT-6 and CFP-10 of substantially larger numbers of individuals with known TB status will be required to address its sensitivity, specificity, and clinical and epidemiological relevance and to determine the most appropriate cutoff between positive and negative reactivities by this assay.

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FIG. 2. Scattergram showing the correlation between CD69 expression on CD4+ T lymphocytes in whole-blood samples stimulated with tuberculin and the TST induration diameter for groups of healthy individuals with a negative TST reaction (group A; open circles) and a positive TST reaction (group B; closed circles).
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