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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 assessment of the cell-mediated immune response to a tuberculin. Although TST has been the "gold standard" for diagnostic screening and the detection of new or asymptomatic TB, it has a number of drawbacks, including the need for a return visit to allow reading of the results, problems with interpretation of the test results due to cross-reactivity with other mycobacterial species, the booster effect, false-negative results due to intercurrent immunosuppression, and the variability inherent in its application and reading (16). In addition, it is imprecise and can give only a partial assessment of the interrelationships among *M. tuberculosis*, host macrophages, and the surrounding cellular components of the immune system, particularly CD4 T lymphocytes (5, 22).

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Different types of blood tests that offer multiple potential advantages over skin testing have been suggested for the diagnosis of TB (7, 24). In 2001, the in vitro test Quantiferon-TB (QFT), manufactured by Cellestis Limited, Carnegie, Victoria, Australia, which measures the release of gamma interferon (IFN-γ) in whole blood in response to stimulation by PPD, was approved by the Food and Drug Administration (22). It has been shown to provide a specific, sensitive, and rapid method for detecting *M. tuberculosis* infection, discriminating between *M. tuberculosis* and *M. avium* infections, and correlates with the TST reaction in individuals with and without human immunodeficiency virus (HIV) infection (10, 12, 19, 20, 23, 27, 33). As with TST, interpretation of the results differs for each person according to his or her risk for LTBI and the development of TB (19).

Determination of the expression of surface antigens is another method for evaluating the lymphocyte effector function (13). 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 (15). 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 (LTT), 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* at 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 of anti-TB chemotherapy and were diagnostically evaluated at our hospital. The diagnosis of active pulmonary TB was confirmed by isolation of *M. tuberculosis* in culture (class 3, according to the diagnostic standards and the classification of TB in adults and children of the American Thoracic Society [ATS] [3]). Group D consisted of 15 individuals (7 females and 8 males) with clinically inactive TB (class 4, according to ATS [3]). The individuals in group D had a history of active TB that was confirmed by the isolation of *M. tuberculosis* in culture and had completed a previously prescribed course of chemotherapy. TB was clinically inactive during the study.

All healthy individuals (groups A and B) were skin tested with 2 tuberculin units of PPD RT23 (Statens Serum Institut, Copenhagen, Denmark) placed intradermally by the standard (Mantoux) technique (2, 3). All readings were performed by the palpation and ballpoint methods. The interpretive criteria used to determine positive TST reactions for groups of BCG-vaccinated healthy individuals were those of ATS (3). The TST reaction was considered positive when the induration diameter was larger than 15 mm at 48 to 72 h after the injection of 2 tuberculin units of PPD (1). The patients in groups C and D were not skin tested. For individuals in both groups, TB was confirmed by isolation of *M. tuberculosis* in culture.

None of the individuals included in the study was immunocompromised. Exclusion criteria for patients were acute infection or inflammatory responses, chronic medical illnesses known to be accompanied by changes in immune functions, neurological disorders, and alcohol and/or drug abuse. Patients receiving immunosuppressive therapy and those with serious medical illnesses were ineligible for study participation.

All subjects who were screened for the study were included. All patients gave written consent to participate in the study. The study was approved by the national Medical Ethics Committee.

QFT. Ten milliliters of blood was collected from all the participants and placed into a heparinized tube. Whole blood was stimulated with PPD (CLS, Biosciences, Parkville, Victoria, Australia), according to the instructions of the manufacturer, and 18 h later the amount of IFN-γ in plasma was measured by QFT. The PPD IFN-γ-specific response was calculated as the percent increase by determination of the amount of IFN-γ produced by PPD-stimulated cells (with subtraction of the background response of cells incubated with saline only) divided by the amount of IFN-γ produced by mitogen-stimulated cells (also with subtraction of the background response).

LLT. Mononuclear cell suspensions at a concentration of 10^6 viable cells per ml were prepared by Ficoll-Hypaque gradient centrifugation from heparinized venous blood by the standard procedure. Triplicate cultures were set up in 96-well flat-bottom microtiter plates (Costar, Cambridge, Mass.) in a volume of 100 μl, and 50 μl of PPD or an equal volume of lymphocyte culture medium (RPMI 1640 medium; Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% human 0 serum as negative culture controls was added at the time of culture initiation. For LTT, the concentration of PPD RT23 (Statens Serum Institut) was 15.6 μg/ml. Cell cultures were incubated at 37°C in a humidified 95% air–5% CO_2 atmosphere for 7 days. The cells were labeled with 37 kBq of [*methyl*-^{3}H]thymidine (specific activity, 3.1 kBq/mmol; Dupont, NEN Research Products, Boston, Mass.) in 50 μl of lymphocyte culture medium 18 h before the cells were harvested with a semiautomatic cell harvester (Skatron, Lier, Norway). The incorporation of [^{3}H]thymidine was determined by liquid scintillation counting (WALLAC 1410; Pharmacia, Uppsala, Sweden). The results are shown as the mean counts per minute for the triplicate samples.

Flow cytometry. A volume of 500 μl of whole blood was incubated with 50 μl of tuberculin PPD (CLS, Biosciences). The samples were incubated at 37°C in a humidified 95% air–5% CO_2 atmosphere for 18 to 24 h. After incubation, fluorochrome-conjugated monoclonal antibodies, CD4 (fluorescein isothiocyanate), and CD69 (phycoerythrin), all purchased from Becton Dickinson (San Jose, Calif.), were added to 40 μl of whole blood to stain the cells, according to the recommendations of the manufacturer. All samples were analyzed with a FacSort flow cytometer (Becton Dickinson) with CellQuest software (Becton Dickinson) for data analysis. The analysis was performed with the scatter gates set on the lymphocyte fraction. At least 10,000 lymphocytes were analyzed in each test. The results were expressed as the difference between the percentage of CD4/CD69-positive cells stimulated with PPD (CLS, Biosciences) and the percentage of the control cells stimulated with saline only.

Statistical analysis. One-way analysis of variance was used to compare the mean CD69 antigen levels for the individuals from the different groups. For groups A and B, the expression of CD69 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 LTT 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 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 C), 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

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	47.6 ± 12.4	47.3 ± 11.8	47.3 ± 12.8
Range	28–69	30–73	37–67	29–72
M. tuberculosis infection status	TST negative	TST positive	Culture-proven TB	Culture-proven TB
% $CD4^+$ T lymphocytes with CD69 expression				
Mean	3.8	15.5	16.2	10.5
SD	4.3	7.2	7.3	7.4
QFT result (%)				
Mean	10.4	92.9	26.5	46.9
SD	12.9	107.7	18.1	37.4
LTT result (cpm)				
Mean	575	2,500	6,336	3,242
SD	819	2,063	4,538	1,830

individuals (groups A and B) correlated significantly with the TST induration diameter (Spearman rho = 0.783; $P < 0.001$) (Fig. 2).

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 = 0.613; $P < 0.001$).

In vitro synthesis of IFN-γ. Patients with active TB (group C), those with clinically inactive TB (group D), and healthy individuals with a positive TST reaction (group B) produced significantly higher concentrations of IFN-γ in vitro in response to tuberculin than healthy individuals with a negative TST reaction (A) ($F = 4.56$; $P = 0.007$) (Fig. 1, upper right panel). The mean concentration of IFN-γ in the group of patients with active TB (group C) was lower than that in the group of healthy individuals with a positive TST reaction (group B).

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 PPD. 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

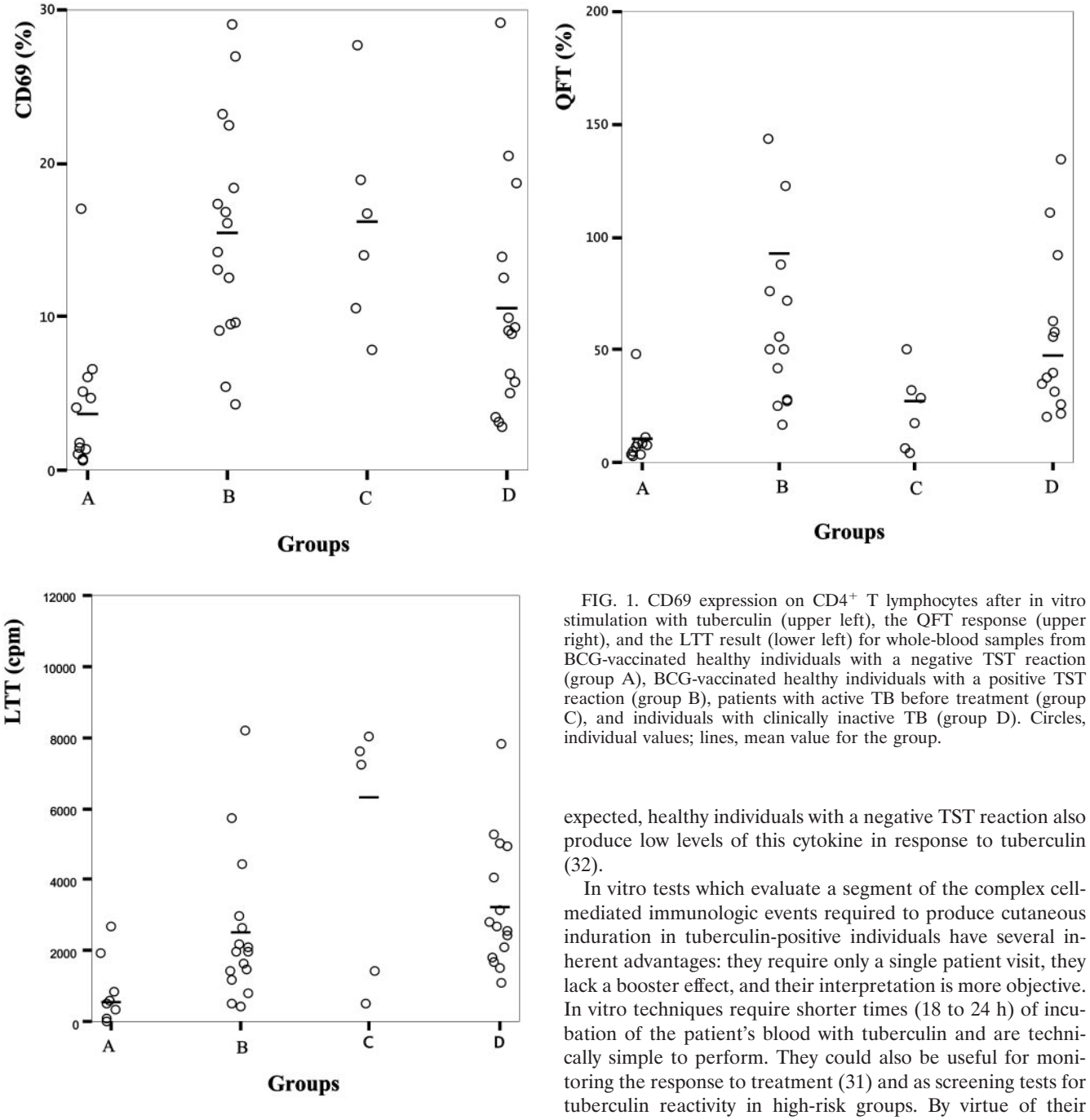

FIG. 1. CD69 expression on $CD4^+$ T lymphocytes after in vitro stimulation with tuberculin (upper left), the QFT response (upper right), and the LTT result (lower left) for whole-blood samples from BCG-vaccinated healthy individuals with a negative TST reaction (group A), BCG-vaccinated healthy individuals with a positive TST reaction (group B), patients with active TB before treatment (group C), and individuals with clinically inactive TB (group D). Circles, individual values; lines, mean value for the group.

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 quantitate 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

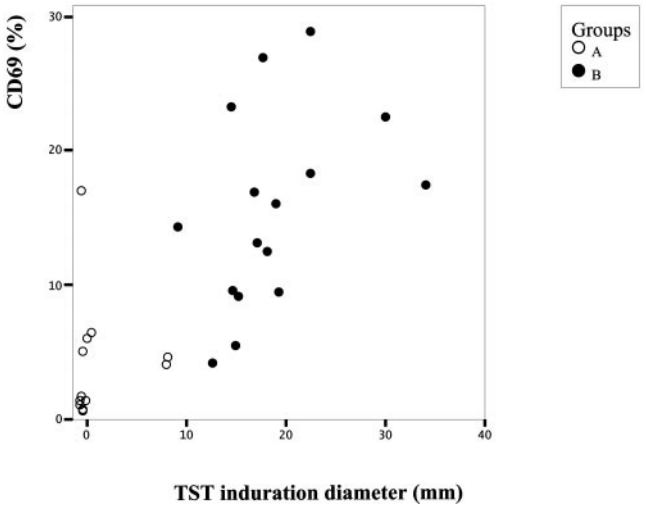

FIG. 2. Scattergram showing the correlation between CD69 expression on $CD4^+$ T lymphocytes in whole-blood samples stimulated with tuberculin and the TST induration diameter for groups of healthy individuals with a negative TST reaction (group A; open circles) and a positive TST reaction (group B; closed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

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 artifactual 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 (17) and CFP-10 (9) were evaluated and found to differentiate individuals infected with *M. tuberculosis* from controls infected with other mycobacteria with high sensitivities and specificities.

The present study has some methodological limitations. Due to the small number of subjects included in the study, the sensitivity and specificity of the test have not been determined. Further studies aimed at assessing its sensitivity, specificity, and clinical and epidemiological utilities for the wider population are warranted. Whether the assay is more sensitive for the detection of individuals with a false-negative skin test reaction is one potential advantage that should be determined. The measurement of CD69 on $CD4^+$ T lymphocytes after in vitro stimulation of substantially larger numbers of individuals with known TB status will be required to address these issues 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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