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Preliminary Evidence of Natural Resistance to *Mycobacterium bovis* (BCG) in Lepromatous Leprosy[1]

Jean-Louis Stach, Georgette Delgado, Michel Strobel, Jacques Millan, and Philippe H. Lagrange[2]

One of the most characteristic features of leprosy is the bipolar partition of patients. Some of them, classified as tuberculoid (TT), vigorously fight *Mycobacterium leprae*, preventing its growth and dissemination throughout the body. Others, known as lepromatous (LL), do not protect themselves, allowing the bacilli to grow and invade the tissues. Since *M. leprae* is an obligate intracellular parasite living in macrophages, it has been hypothesized that there is some kind of primary deficiency in macrophages from LL patients ([1-3]). Studies in favor of this hypothesis have not been confirmed, and macrophages from LL patients are now believed not to be deficient ([7]) according to the usual criteria of testing macrophage activation, which still need a more accurate definition. On the other hand, fitting into the Mackaness model of infectious disease, there is general agreement that in leprosy LL patients are not protected because of a lack of specific immunity against *M. leprae* ([19]). Experimental studies of infectious diseases produced by intracellular multiplying microorganisms have shown that this simple assessment, emphasizing a single role of the specific immune response, was incomplete ([19]) and that overall acquired resistance to an infectious agent must be considered as the result of two phenomena. The first one is now described as the "natural resistance" that is demonstrated during the early phase of the infection and is experimentally evoked after an intravenous (IV) injection of a low dose of pathogens ([20]). The second one involves the specific "immune resistance" which occurs later and is experimentally demonstrated by the immune granuloma following local injections ([19]). Natural resistance to a given parasite is genetically controlled and is often nonspecific regarding the infective agent ([20]). For instance, in mice natural resistance to *M. bovis*, strain BCG, is governed by a single, autosomal, dominant gene located on chromosome 1 ([10]). This gene, named Bcg, is closely linked or probably identical to gene(s) controlling natural resistance to two unrelated microorganisms, namely *Leishmania donovani* (*Lsh* gene) and *Salmonella typhimurium* (*Ity* gene) ([20]).

More recently, the Bcg gene has been shown also to control resistance to *M. lepraemurium*, the causative agent of murine leprosy ([4, 21]). Experimental evidence showed that resident peritoneal macrophages were able to express the natural resistance to BCG as tested by *in vivo* as well as by *in vitro* tests ([22, 23]). During these studies, *in vitro* macrophage inhibition of BCG ^{3}H uracil incorporation appeared to be an accurate test for the Bcg gene ([23]). This new opportunity will lead to further studies on phenotypic expression mechanisms of the Bcg gene in mice ([22]) and might allow feasible explorations of the natural resistance to mycobacterial infections in humans, which otherwise would have been difficult for many obvious reasons. Furthermore, since natural resistance to *M. lepraemurium* has been shown to be controlled by the Bcg gene in mice ([23]), it was worthwhile to evaluate if natural resistance to BCG in humans might be linked to resistance or susceptibility to *M. leprae* in leprosy patients. This prompted us to undertake preliminary comparative

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[2] J.-L. Stach, M.D.; G. Delgado, B.S.; and M. Strobel, M.D., Laboratoire d'Immunologie Cellulaire, Institut Pasteur de Dakar, Dakar, Senegal. J. Millan, M.D., Institut de Leprologie, Dakar, Senegal. P. H. Lagrange, M.D., Laboratoire d'Immunobiologie du BCG, Departement de Physiopathologie Experimentale, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris, France. Reprint requests to Dr. Lagrange.

studies on macrophage-induced inhibition of ^{3}H uracil uptake into BCG in lepromatous and tuberculoid patients, using a test described by Rook and Rainbow ([18]). The present report shows somehow unexpected results: LL patients' blood monocytes in culture being more "naturally resistant" to BCG than monocytes from TT patients.

MATERIALS AND METHODS

Patients. All tested patients were from the Institut de Léprologie in Dakar (Dr. Millan), and were typed according to the clinical, bacteriological, immunological, and histological criteria of the Ridley and Jopling classification ([17]). Their ages varied from 18 to 60 years. They have been studied and coded by pairs (one LL and one TT). Their cells were treated strictly in the same way throughout the study. To avoid any eventual role of chemotherapeutic agents, only newly detected patients, free of any treatment, were included in this study.

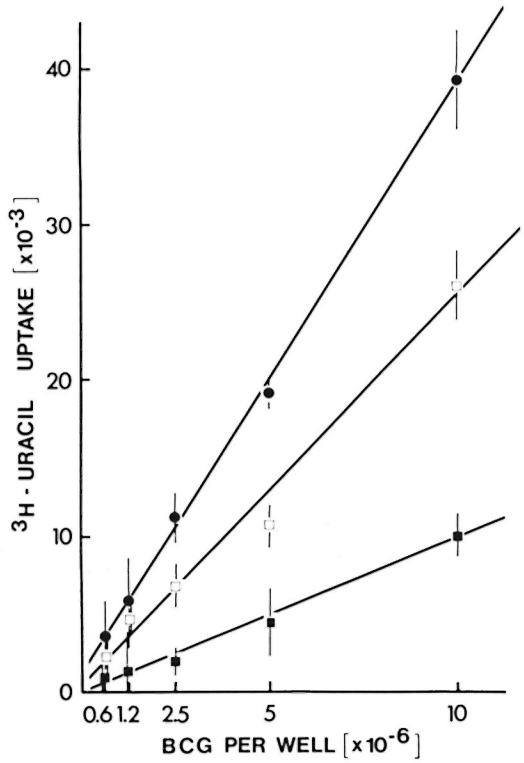
***M. bovis*, strain bacille Calmette-Guerin (BCG).** The BCG used was the Pasteur strain (1173P2) regularly grown in liquid Sauton medium at the Institut Pasteur de Dakar in order to produce commercially available vaccine. Six- to ten-day culture tubes were shaken and centrifuged at 1500 rpm for 5 min to get rid of large clumps. The single organism suspension was adjusted to the desired concentration in cell culture medium after determination of the number of bacilli per ml in an appropriate counting chamber.

Collection of macrophages. Monocytes were obtained from 20 ml of blood by venipuncture taken on heparin (10 IU/ml) (Liquemine, Roche Lab.). Mononuclear cells were isolated on a Ficoll-Hypaque (Pharmacia) gradient according to the usual methodology, washed twice in Hanks' buffered salt solution (HBSS) and suspended at 4×10^6 cells/ml in buffered RPMI medium 1640 (Flow Laboratories) supplemented with 2 mM L-glutamine (Gibco) and 10% pooled heat-inactivated human AB serum from normal subjects. The cells were allowed to adhere on a 35 mm plastic Petri dish for 1 hr at 37°C in a 5% CO_2 incubator. After vigorous agitation, nonadherent cells were discarded. This procedure was repeated twice after addition of prewarmed culture medium. Adherent cells, thereafter named macrophages, were removed after a 30 min treatment with EDTA added to the medium (3 mg/ml). As judged by May-Grünwald Giemsa (MGG) staining, they were 95% pure monocytes-macrophages. Recovered cells were washed 3 times with cold HBSS, and once with culture medium, then adjusted to 2×10^6 cells/ml and put into 96 well microtiter culture plates (100 μl per well) which were placed for 1 hr at 37°C in a 5% CO_2 incubator. After this incubation period, monolayers were washed once again before performing the assay. At this stage, control wells showed almost pure populations of macrophages (MGG staining).

Measurement of ^{3}H uracil incorporation into BCG. The test was performed according to Rook and Rainbow ([18]) with some modifications. Since the rate of ^{3}H uracil incorporation depends largely on the strain of BCG and its physiological state ([22]) and since low numbers of BCG were used to infect macrophages resulting in very low rate of ^{3}H uracil incorporation into BCG, higher infectious ratios (numbers of BCG per macrophage) ranging from 3/1 to 50/1 were used. Microtiter wells, as previously prepared, were washed once and the culture medium over the cell monolayer was replaced by 100 μl of the appropriate BCG suspension to achieve the infecting ratio indicated above. Two controls were included, consisting of wells containing macrophage monolayers overlaid with medium alone, and wells free of macrophages but seeded with 100 μl of the bacilli suspensions.

Plates were incubated at 37°C in a 5% CO_2 incubator for 4 days. Saponin (0.1% final concentration) was added to each well and the plates were incubated 1 hr further to lyse macrophages. Finally 10 μl of ^{3}H uracil containing 1 μCi (51 μCi/nmol, Amersham, TRK 408) was added and, after a further 24 hr incubation, the content of each well was recovered with a Skatron cell harvester (wells flooded with saline, TCA 5% and pure methanol). TCA precipitable radioactivity on Whatman filters was measured in counts per minute (cpm) for 1 min in 5 ml scintillation liquid (Biofluor, New England Nuclear) in a liquid scintillation counter (Intertechnique, France).

All the determinations were made in quadruplicate samples. No antibiotic was added



THE FIGURE. *In vitro* effect of macrophage-BCG interaction on ^{3}H uracil incorporation into bacilli. Monolayers of macrophages from tuberculoid leprosy Patient 225 (□—□) and lepromatous leprosy Patient 226 (■—■) containing 2.10^5 cells/well were prepared in microtiter plates as indicated in Materials and Methods. Washed monolayers of cells were overlaid with 100 μl of BCG suspension (in cell culture medium) containing the indicated numbers of BCG/well. Control BCG wells (●—●) were seeded with 100 μl of the same suspension without macrophages. Control macrophages contained macrophages alone. (Mean of quadruplicate ± standard error of the mean.)

THE TABLE. *Comparison of the inhibition index of macrophages*[a] *from LL and TT patients.*[b]

		LL PATIENTS					
		233	226	228	221	219	214
TT PATIENTS	232	4.8 / 0.9				5.7 / 2.6	
	229		1.4 / 1		1.9 / 1		
	225		3.7 / 1.1				5 / 1.5
	227	3.1 / 2.6		8.2 / 4.8		2.1 / 0.9	
	222				1.2 / 1.1		
	218					6.5 / 3.1	
	217		7 / 3				4.1 / 3.6

[a] Results were given as inhibition index calculated as indicated in Materials and Methods. Each square represents one experiment. The upper number, on the right, is the inhibition index of the LL patient; the lower number, on the left, is the inhibition index of the TT paired patient.

[b] Macrophages from one LL and one TT patient were tested simultaneously for their inhibitory effect on BCG metabolism.

at any stage. Results obtained from different experiments were expressed graphically as cpm of ^{3}H uracil uptake of the appropriate BCG suspension used. Since it was always found that cpm were a function of the BCG numbers added per well, it was possible to draw regression lines. Therefore, results of all experiments were expressed in the following way: a regression line average cpm incorporated per well was drawn as a function of the infecting ratio using the method of least squares. The slope of that straight line was calculated for BCG alone and BCG added to macrophages. Then, an inhibition index was defined for each patient's macrophages as the ratio between the slope of the regression line for BCG alone and the slope of the regression line for BCG in the presence of macrophages.

RESULTS

Results of a typical experiment are shown graphically in The Figure. ^{3}H Uracil incorporation into BCG alone (control) after four days of culture showed a linear relationship with the initial number of BCG seeded per well; similar linear relationships were also observed for BCG added to macrophage monolayers. Counts in the wells containing both macrophages and BCG are due solely to ^{3}H uracil incorporation into BCG, since macrophages alone did not incorporate any appreciable radioactivity in the conditions of the test. For instance, incorporation of ^{3}H uracil into control macrophages was 218 ± 106 and 311 ± 92, respectively, for Patients 225 (tuberculoid) and 226 (lepro-

matous). As already mentioned, results in The Figure showed that it was possible to draw a regression line for ^{3}H uracil incorporation as a function of the numbers of BCG added per well (or infecting ratio). The macrophage action upon BCG uracil uptake appears as a decrease in the slope of that straight line. Therefore, results of all experiments were expressed as an inhibition index of macrophages on ^{3}H uracil incorporation by BCG. Calculated slopes of the regression line were 739 for BCG alone, 652 for BCG plus macrophages from Patients 225 (tuberculoid leprosy) and 201 with macrophages from Patient 226 (lepromatous leprosy). The inhibition indexes were then 1.1 (739/652) for Patient 225 macrophages and 3.7 for Patient 226 macrophages. The inhibition index is then directly proportional to the inhibitory effect of macrophages.

The Table shows that, in all of the experiments, macrophages from LL patients always expressed an inhibition index which was greater than macrophages from tuberculoid patients. Some patients were tested two or three times in different combinations. As can be observed, the inhibition index value for a single LL or TT patient varied from one experiment to another, so the index cannot yet be considered as characteristic of the patient. The comparisons between two persons are only accurate, in the conditions used, if they are tested during the same experiment. Nevertheless, it must be stressed that in any of the tested combinations of patients, LL macrophages always expressed inhibition indexes greater than those of TT patients. Thus, this tends to indicate that any LL macrophage is always more aggressive against BCG than a macrophage from any TT subject.

DISCUSSION

Human blood monocytes in culture have been shown to have a potent inhibitory effect upon BCG metabolism as judged by the level of incorporation of ^{3}H uracil, a RNA precursor, confirming preceding results obtained by Rook and Rainbow ([18]). This precursor was not incorporated by macrophages in appreciable amounts under the conditions used. Therefore, ^{3}H uracil can be used as a marker of the BCG metabolism which appears to be depressed in the presence of macrophages. Using this test, new evidence showed that macrophages from LL patients are not intrinsically deficient compared to TT macrophages. On the contrary, they appeared to be more inhibitory on BCG metabolism than did TT macrophages.

It recently has been shown in a mouse model that macrophage inhibition of ^{3}H uracil incorporation was an accurate *in vitro* test of natural resistance to BCG. Non-induced, resident, peritoneal macrophages explanted from a naturally resistant, C3H strain of mice as defined *in vivo* ([10]), were more inhibitory for BCG ^{3}H uracil incorporation than resident peritoneal macrophages from a naturally susceptible C57BL/6 strain ([23]). The experiments reported here were not performed under these conditions in which non-induced resident macrophages from normal mice were used. This was not done because it has been shown that high lysosomal activity is found in the macrophages of these chronically infected patients ([25]). Additionally, the blood-derived macrophages used in the present study might be a part of a subpopulation, or cells in a physiological state which might be very different from the normal resident macrophages of mice. For example, they might be immune-activated or immune-suppressed macrophages in the different human populations tested. It is reasonable to speculate, but not proved, that non-adherent-depleted monolayers of macrophages used in this work were not contaminated with T cells. There is also evidence that isolated macrophages lose their immune activation state when they are separated from the influence of T cells ([16]). Since TT patients are known to have a strong cellular immune response, while the LL response is depressed, one would have expected TT macrophages to be activated and LL macrophages suppressed. Since the opposite occurred, in repeated experiments, it can be considered that the respective immune response of the patients did not have any influence on the results.

If we assume that, as in the mouse model, the test is an accurate *in vitro* test for natural resistance, we must consider LL patients as naturally resistant to BCG and TT patients as susceptible. A mouse naturally resistant to BCG has on its chromosome 1 the Bcg^{r} allele of the Bcg gene. This allele makes the mouse able to inhibit the growth of BCG

and is also associated with a higher resistance to *M. lepraemurium* infection ([4, 12, 21]). If one transposes the results from mouse studies to humans, it can be speculated that LL patients have an equivalent of the Bcg^r allele, while TT patients bear the Bcg^s allele. LL patients, therefore, must be naturally resistant to *M. leprae* and TT patients, naturally susceptible. Obviously there is an apparent paradox here.

It is hard to understand how a subject resistant to *M. leprae* can let himself be invaded by the bacilli. However, careful observations of murine experimental data show that the situation is not so paradoxical ([13]). A mouse is defined as naturally resistant to BCG if there is no growth or limited growth of bacilli in the spleen three weeks after IV injection of a small dose (10^4/mouse) of dispersed bacilli ([10]). According to this protocol, C3H mice were classified as a naturally resistant strain; C57BL/6 mice being naturally susceptible. C3H mice were also described ([4]) as resistant (according to the survival time) to 1×10^8 *M. lepraemurium* injected IV. But if C3H mice were inoculated subcutaneously with 1×10^7 *M. lepraemurium,* they behave as susceptible, allowing the bacilli to grow and to spread freely into the tissues ([5]). On the contrary, C57BL/6 mice injected subcutaneously with the same inoculum of *M. lepraemurium* behave as resistant, limiting bacillary invasion through the formation of a local immune granuloma ([5, 12]). So according to the route, the inoculum dose, and the absence or presence of immunopathological reactions, the same strain of mice can be classified as either resistant or susceptible ([13]). However, this latter classification of resistance or susceptibility is different from the former, i.e., natural resistance or susceptibility, since it involved the presence or the absence of a specific acquired immune response ([13]).

The pattern might be the same in humans, where LL would be analogous to C3H and TT analogous to C57BL/6 mice. Thus it is necessary to consider resistance to leprosy as a two-step phenomenon. A first line of defense would be the natural resistance involved in the eradication of a small infectious inoculum, an inoculum which alone does not elicit an immune response or, even if it does elicit an immune response, the response might be involved in the induction of tolerance. The second line of defense would be resistance, operating through the classical scheme: sensitization of T cells which recruit new cells (granuloma formation) and activate effector cells. In spite of controversial results, *M. leprae* seems to resist the normal macrophage process of total bacterial destruction ([1, 7, 19]). Thus, the only way to limit the infectious process of dissemination must be the formation of an immune granuloma ([13]).

As described in the mouse model, C3H mice in spite of or because of their natural resistance do not build an accurate immune response ([12, 14]). Whether or not the failure to produce the right type of immune response is a consequence of high natural resistance is an intriguing possibility ([15]). For instance, one can imagine that macrophages in charge of the natural resistance are of a special subpopulation of macrophages, efficient in phagocytosis but weak in the antigen presentation function (through a lack of Ia surface antigens for instance). As shown by Gorczynski and MacRae ([9]) with *Leishmania tropica* in mice, such a macrophage subpopulation being predominant in a naturally resistant individual would result in a poor immune response. In this respect, the studies by Hirschberg ([11]) showing a failure of antigen presentation by LL macrophages might fit with this explanation. However recent evidence from Stoner, *et al.* ([24]) showed that blood-derived macrophages from HLA-DR matched lepromatous leprosy patients were still able to present specific *M. leprae* antigens to blood-derived lymphocytes from responder control siblings. This implies another mechanism for the unresponsiveness.

The results presented in this preliminary report are in agreement with the results obtained by Yamashita, *et al.* ([25]), showing high levels of lysozyme activity in macrophages from lepromatous leprosy nodules. It has already been shown that lysosomal enzyme levels rose when monocytes were cultured *in vitro* ([8]) but decreased when lymphokines, such as macrophage migration inhibitory factor (MIF), were added ([6]). These observations support our point of view that blood-derived macrophages in LL nodules or LL blood monocytes in culture behave

as naturally more active macrophages than those from TT patients.

The assay described in this study, with its preliminary results, opens a new way to evaluate the natural resistance of an individual to mycobacterial infection and might represent, after repeated independent studies, an accurate test for the detection of high-risk, nonresponsive subjects in leprosy or in tuberculosis.

SUMMARY

An assay system has been developed based on radiometric quantification of ^{3}H uracil incorporation into viable BCG in the absence or presence of blood monocytes in cultures from untreated lepromatous (LL) or tuberculoid (TT) leprosy patients. ^{3}H Uracil incorporation into BCG was inhibited when the bacilli were cultivated in the presence of blood-derived macrophages in culture for four days, and that inhibition was always greater with macrophages harvested from LL patients compared to TT patients. The reasons for such an observed difference in humans are discussed according to our knowledge obtained in murine models of mycobacterial infections.

RESUMEN

Se desarrolló un método para medir la incorporación de uracilo-^{3}H por bacilos BCG viables cultivados en ausencia o en presencia de monocitos provinientes de pacientes con lepra lepromatosa no tratada (LL) o de pacientes con lepra tuberculoide (TT). La incorporación del uracilo-^{3}H por el BCG fue inhibida en presencia de monocitos sanguíneos cultivados por 4 días, pero la inhibición fue siempre mayor en presencia de macrófagos provinientes de pacientes LL que en presencia de macrófagos derivados de pacientes TT. Se discuten las razones de este diferente comportamiento en humanos en base a lo que se sabe de las infecciones micobacterianas en modelos murinos.

RÉSUMÉ

On a développé un système d'évaluation basé sur la quantification radiométrique de l'incorporation du radical uracil marqué à l'^{3}H par le BCG viable en absence ou en présence de monocytes sanguins provenant de malades de lèpre lépromateuse (LL) ou (TT), et mis en culture. L'incorporation d'uracil ^{3}H dans le BCG a été inhibé lorsque les bacilles étaient cultivés en présence de macrophages dérivés du sang et cultivés pendant quatre jours; cette inhibition était toujours plus prononcée lorsqu'on utilisait des macrophages recueillis à partir de malades LL, que quand on faisait usage de macrophages prélevés chez des malades TT. Les raisons de cette différence chez l'homme sont discutées, à la lumière des connaissances obtenues dans les modèles murins d'infections bactériennes.

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