

Natural Cell-Mediated Cytotoxicity in Normal Human Peripheral Blood Lymphocytes and Its *in vitro* Boosting with BCG

Rosemonde Mandeville, François-Mambo Sombo, and Normand Rocheleau

Immunology Research Center, Armand-Frappier Institute, C.P. 100, Laval-des-Rapides, Quebec, Canada H 7N 4Z3

Summary. Natural cell-mediated cytotoxicity (NCMC) against K-562 human erythroleukemic cells was monitored in an overnight chromium release assay using normal human peripheral blood lymphocytes (PBL) as effector cells. Two hundred and ten normal individuals were tested from 3 to 24 times over a period of 3 years. The level of NCMC was shown to vary from 4% to 46% lysis at an effector-to-target cell ratio of 5/1; males had higher levels of activity than females ($P < 0.001$). A group of individuals with low natural killer (NK) cell activity (below the 90% tolerance limit) was identified in replicate experiments and 60% of them were young women (ages 20–39). *In vitro* boosting of NK activity with *Bacillus Calmette-Guérin* (BCG) was also studied; overall, 56% of normal individuals responded positively to BCG. There was a significant ($P < 0.0001$) correlation between the unstimulated level of NCMC and the *in vitro* boosting with BCG, as 63% of individuals with a normal level of NK activity could be boosted as against only 19% of persons with low NK activity. We have also established the *in vivo* relevance of this *in vitro* test by determining the degree of correlation between responses to *in vitro* boosting with BCG and a positive or negative reaction in a hypersensitivity skin test using 5 IU of PPD (purified protein derivative of BCG). Our results indicate that NCMC is an individual trait that varies little under physiological conditions, and that the response to BCG is a characteristic property of the effector lymphocyte, depending primarily on the unstimulated level of NCMC.

Introduction

It has been shown that human peripheral blood lymphocytes (PBL) and cells recovered from various lymphohematopoietic organs of experimental animals can spontaneously lyse many types of tumour cell lines in the absence of any previous sensitization. This activity has been termed natural cell-mediated cytotoxicity (NCMC) and is mediated by natural killer (NK) cells [reviewed in 5, 15]. These NK cells appear to have lymphocyte morphology [31], arise *de novo* in the bone marrow but lack the characteristic markers of mature T or B cells. The precise biological significance of NCMC is at present unclear, although NK cells have been proposed as the first line of cell-mediated defense against newly arising malignant cells and against viruses. In view of this suggested role of NK cells, it is important to understand the factors that influence NK activity. A variety of factors are known to alter

NK activity: drug treatment with hydrocortisone [5], estradiol [29], cyclic nucleotides, and prostaglandins inhibit NK activity, while interferon and interferon inducers, bacterial adjuvants, tumor cells, and viruses augment it. Elevated NK activity has also been documented in PBL from chronic alcoholic human subjects [27]. NCMC has been shown to be depressed in a number of disease states: malignancy, severe combined immunodeficiency, autoimmune diseases, multiple sclerosis, infectious mononucleosis, and Chediak-Higashi syndrome [1, 7, 9, 13, 14, 18, 21, 22, 24, 30].

Since the extent of NCMC depression or boosting is often estimated and expressed only in comparison with control values, it is of primary importance to know the normal range of NK activity in humans for meaningful evaluation of lymphocyte reactivity of a particular patient. Surprisingly, only a limited number of reports of NCMC levels in normal individuals have been published, and in each case only a small number of individuals were evaluated [16, 19, 20, 23].

The purpose of the present communication is (a) to establish the reproducibility of results in the measurements of NCMC; (b) to evaluate the mean levels of activity in a large group of normal donors to establish baseline figures for NCMC levels; (c) to compare NK activity in sex and age subsets of the normal population; (d) to investigate the relative ability of individuals to respond to BCG *in vitro*; and (e) to establish the *in vivo* relevance of the response to BCG. Although BCG has been widely used in the immunotherapy of cancer, there is very little information on the effects of BCG on various immune parameters or on the mechanism(s) by which BCG exerts its therapeutic effects in cancer patients.

Materials and Methods

Subjects Studied. Peripheral blood lymphocytes (PBL) were collected in heparinized vacutainer tubes from 210 normal adults of both sexes, aged 20–72. All were in apparent good health and took no medication during the study period. A small questionnaire was given to each person to assess their smoking habits, alcohol and drug consumption (aspirin or corticosteroids), and recent medical history. Additional questions were asked of women in the study regarding the time in menstrual cycle, contraceptive usage, and any surgery that could affect their hormonal balance, e.g., hysterectomy, ovariectomy, or thyroidectomy.

Effector Cell Isolation. Immediately after collection, 100 mg carbonyl-iron was added to each 10 ml blood and the mixture

was incubated for 45 min at 37° C with constant shaking. The cells were then layered on a Ficoll-Hypaque density gradient (Pharmacia, Uppsala-Nyegaard Oslo, Norway). Lymphocytes were collected at the interface, washed three times, counted, and resuspended to a concentration of 2×10^6 viable cells/ml in RPMI-1640, containing 25 mM HEPES, 4 mM L-glutamine, antibiotics penicillin 100 IU/ml and streptomycin 100 µg/ml and 10% heat-inactivated fetal calf serum (FCS).

Target Cells. K-562, an erythroleukemic cell line derived from the pleural effusion of a patient with chronic myelogenous leukemia in blast crisis [10] was kindly supplied by Dr. M. G. Baines, McGill University, and was used as the target cell. It was maintained as a stationary suspension culture in RPMI-1640 supplemented with 10% FCS and antibiotics. K-562 cells were labeled by mixing 1×10^6 cells with 0.1 ml culture medium containing 100 µCi⁵¹Cr (sodium chromate, Frost, specific activity 2 mCi/mg) and incubating the mixture at 37° C for 45 min. The labeled cells were then washed three times (200 g for 2 min) with cold RPMI-1640. The viability was checked by trypan blue dye exclusion and cells were resuspended to a concentration of 1×10^5 /ml and maintained at room temperature until required.

Cytotoxicity Assay. The technique consisted of an overnight (16–18 h) ⁵¹Cr-release assay. Variable numbers of lymphocytes along with 1×10^4 chromium-labeled target cells were placed in round-bottom microplates (Limbro, New Haven, Conn., USA). The microplates were centrifuged (200 g for 10 min) before and after incubation at 37° C in a humidified atmosphere containing 5% CO₂. Control incubations were performed in the absence of effector cells. The label released from the lysed targets was counted and the percent lysis in the assay was calculated from the following formula:

$$\% \text{ lysis} = \frac{\text{cpm (test)} - \text{cpm (spont)}}{\text{cpm (max)} - \text{cpm (medium)}} \times 100.$$

The cpm (spont) was determined by counting the radioactivity of labeled target-cell suspension, while cpm (max) was the radioactivity of resuspended labeled target cells.

In vitro Boosting with BCG. The same lyophilized preparation of the Montreal strain of BCG was used throughout the study. It was prepared at the Institut Armand-Frappier and contained approximately 2.1×10^8 viable bacilli per ampoule. It was diluted in RPMI-1640 just before use, to final concentrations of 10^2 , 10^4 , 10^6 , and 10^8 bacilli/ml, and 0.1 ml of each dilution was added directly to the test system at the same time as lymphocytes were added to target cells. The percentage of BCG-induced specific boosting of cytotoxicity was calculated by the following formula for triplicate samples:

$$\% \text{ specific boosting} =$$

$$\frac{\text{cpm (BCG-treated cells)} - \text{cpm (untreated cells)}}{\text{cpm (max)} - \text{cpm (untreated cells)}}.$$

Hypersensitivity Test to PPD. The hypersensitivity to PPD (purified protein derivative of BCG) of each individual was ascertained as previously described [11]. Briefly, 5 IU of PPD (Connaught Labs) was injected ID into the forearm and reactivity was read after 72 h. Only indurations greater than 4 mm were considered to be positive. Only individuals known

to have been previously vaccinated with BCG were selected for this study.

Statistical Analyses. Differences between the distribution of NCMC in males and females were compared for significance using Student's *t*-test and Wilcoxon's rank sum test [4]. The normal range was calculated by the one-sided 90% expectation tolerance limit using Tuckey's method [33] based on results obtained for the male population. Controls falling into the 90% tolerance limit (normal range) were identified as having 'normal NK activity', while those falling below that limit (15% lysis) were identified as 'low NK activity'. Comparisons between different groups of controls were made using 2×2 contingency tables.

Results

1. Reproducibility of NCMC

A total of 210 normal healthy individuals (aged 20–72) of both sexes were tested for NCMC over a period of 3 years. Figure 1 shows the mean NK activity (\pm standard error) of all of our donors who have been tested from three to 24 times, and illustrates the small degree of variations between measurements. The reproducibility of this assay indicates that NK activity is an individual trait that varies little under physiological conditions and that for each donor the cytotoxicity level is fairly constant. This consistency in activity is maintained over the years and allows the establishment of patterns of

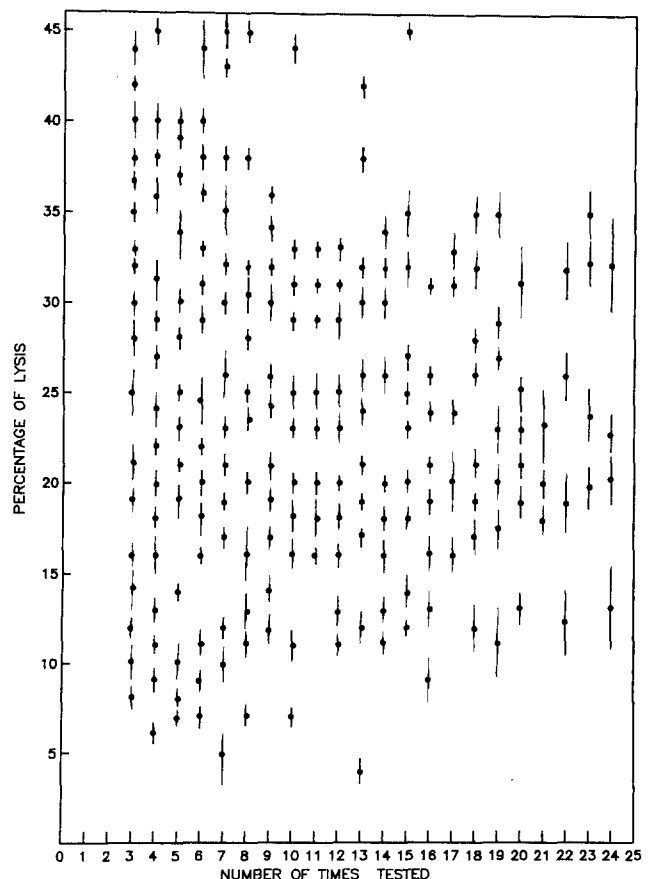


Fig. 1. NK activity of repeatedly tested (3–24 times) normal healthy donors over a period of 3 years. Each donor contributes one value and is represented by the mean (\pm standard deviation) of several tests

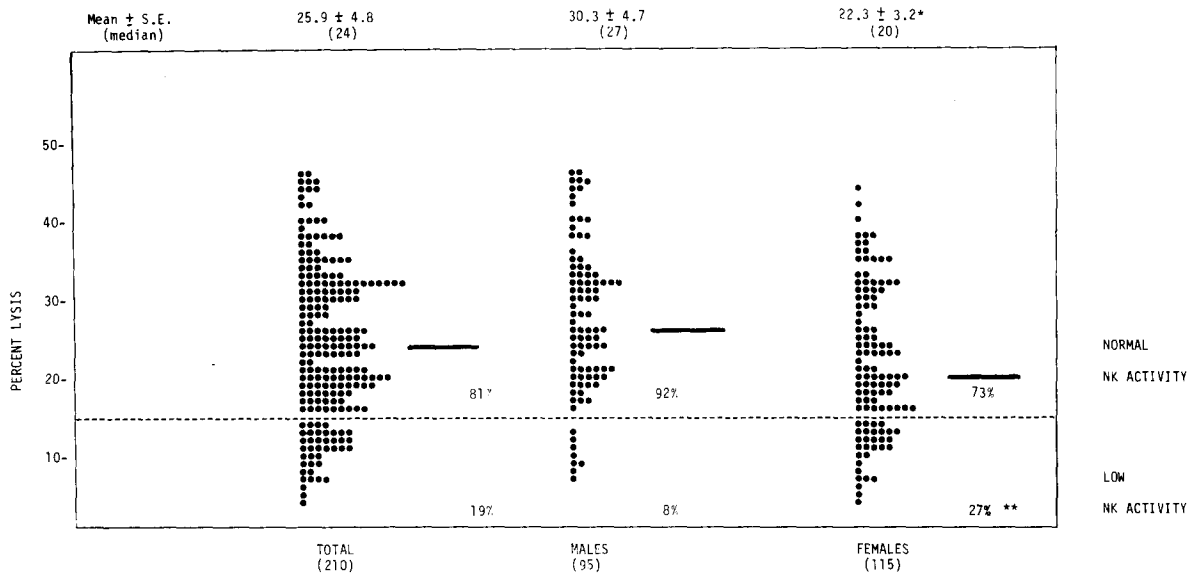


Fig. 2. Distribution of NK activity in 210 normal (ages 20–72) individuals at a E : T ratio of 5 : 1. Each point represents the mean % lysis of an individual when evaluated in repeated experiments. NK cell donors were divided into normal NK activity and low NK activity groups. The cut-off value (---) represents the 90% tolerance limit calculated for the male population. The solid line represents the median for each population. * $P < 0.0004$ when the distribution of NK activity was compared in males and females; ** $P < 0.0001$ when the number and percentage of donors with low NK activity were compared within each sex and between both sexes (Wilcoxon rank-sum test)

normal reactivity in healthy donors; more importantly it could allow comparative studies to determine the effect of disease on NK function.

2. Levels of NCMC Among Healthy Donors

Next, baseline patterns of NK activity were established. For each individual donor the mean percentage of NCMC was calculated. Figure 2 is a summary of this study, clearly showing that the range of NK activity among normal donors varies between 4% and 46% lysis at the E : T ratio of 5 : 1. We then established the normal range of NCMC by calculating the one-sided 90% lower tolerance limit as 15% lysis. Individuals with NCMC levels falling within the normal range were designated as having normal NK activity, while those falling below 15% lysis had low NK activity. The most important observation was that individuals were consistently normal- or consistently low-NK donors. Several factors related to the sources of the blood specimens were next examined, to determine their possible effect on the two different levels of activity.

3. Effect of Sex on NCMC Levels

We found a significant difference in NK activity between male and female donors. Males had significantly higher overall NCMC activity than females ($P < 0.0004$). Furthermore, statistically significantly more females than males fell into the low NK activity group: 27% of females as against 8% of males ($P < 0.0001$, see Fig. 2).

4. Effect of Age on NCMC Levels

In males, age did not play an important role in the expression of NCMC, as the number and percentage of donors with normal and low NK activity was essentially the same in all age groups (Table 1). In females, however, a statistically significantly ($P < 0.05$) higher proportion of young women, 23 of 73 (31%), had low levels of NK activity than of older women

Table 1. Effect of age and sex on the number and percentage of individuals with normal and low NK activity

Age group	Number tested	Normal NK activity (% cytotoxicity) ^a	Low NK activity (% cytotoxicity)
Males			
20–39 years	52	49 (94%)	3 (6%)
40–59 years	26	24 (92%)	2 (8%)
60+ years	17	14 (82%)	3 (18%)
Females			
20–39 years	73	50 (68%)	23 (31%) ^b
40–59 years	20	17 (85%)	3 (15%)
60+ years	22	18 (82%)	4 (18%)

^a % cytotoxicity at E : T ratio 5 : 1

^b When considered as a group, young women (ages 20–39) show a statistically significant difference in the distribution of normal and low NK activity from older women ($P < 0.05$) and males in the same age group ($P < 0.004$)

(15% in women between 40 and 60 and 18% in women aged 60 or more) and of males in the same age group (3 of 52; 6%) ($P < 0.004$). Moreover, among the 38 individuals (male and female) that showed a consistently low level of NCMC, 23 were young females, i.e., 60%. This finding was very surprising, since all individuals tested were overtly 'healthy' donors and suggested a possible hormonal effect on NCMC. However, a thorough analysis of the questionnaires filled out by the female participants in the study failed to reveal any correlations of low NK activity with menopause, time in menstrual cycle, contraceptive intake, or surgery affecting hormonal levels. Quantitative analysis of serum levels of estrogens, growth hormones and thyroid hormones was not performed this study as the effects of sex and age differences on NK activity did not become apparent until most individuals had been tested and thus retesting of all individuals would have been necessary.

Table 2. Effect of in vitro treatment with BCG on NK activity

Treatment	Concentration	% Cytotoxicity in donor ^a			
		A	B	C	D
None	—	50 ± 1.5	40 ± 0.6	30 ± 0.1	32 ± 2.8
BCG	10 ²	61 ± 2.2	56 ± 2.0	41 ± 1.9	50 ± 3.4
(bacilli/ml)	10 ⁴	56 ± 0.5	55 ± 0.9	40 ± 0.2	48 ± 0.7
	10 ⁶	55 ± 3.1	54 ± 1.1	39 ± 2.2	42 ± 0.7
	10 ⁸	27 ± 1.6	36 ± 1.7	18 ± 1.2	22 ± 3.0

^a % cytotoxicity at E:T ratio of 10:1 in four different donors. BCG was added to the test system at the same time as effectors were added to the target cells. Note that for each donor a boosting of NK activity is observed at the 10², 10⁴, and 10⁶ bacilli/ml concentrations, with an inhibition of NK activity occurs at the 10⁸ bacilli/ml concentration

Table 3. Effect of spontaneous level of NCMC on the in vitro NK response to BCG

Population subsets	Number stimulated per number tested	% of population
Normal NK activity	102/160	63%
Low NK activity	6/31	19% ^a
Total	108/191	56%

^a The number and percentage of donors with low NK activity who could be stimulated in vitro with BCG was significantly lower than that of donors with normal NK activity normal responders ($P < 0.0001$)

Table 4. In vivo hypersensitivity tests (PPD) and in vitro boosting (BCG) of NK cells in 128 previously vaccinated individuals

		PPD in vivo	
		(+)	(-)
BCG in vitro	(+)	63	24
BCG in vitro	(-)	18	23

Lymphocytes from 128 donors were tested with BCG in vitro in replicate experiments (for details of technique see *Materials and Methods*). These same donors were tested with 5 IU of PPD (Connaught) and their response was recorded positive (+) when greater than 4 mm induration was measured after 72 h inoculation. Only subjects who had been previously vaccinated with BCG were selected for this study. $P < 0.004$ when the in vitro and in vivo responses were compared by Fisher's exact test. Concordance was evaluated by measuring the gamma variable and was calculated as 0.541 for this study

5. In vitro Boosting with BCG

Addition of BCG to the test system resulted in an increase of NCMC in 56% of the cases studied at the 10², 10⁴, 10⁶ bacilli/ml concentrations, while an inhibition was observed at 10⁸ bacilli/ml (Table 2). The rest of the individuals could not be stimulated at any of the concentrations of BCG used. Quantitatively, a response was considered to be significant only when the percent of specific boosting was greater than 30%.

We then studied the effect of a number of variables on the in vitro response to BCG and found that the reaction frequency

Table 5. In vivo hypersensitivity tests (PPD) and in vitro boosting with BCG in 36 recently vaccinated individuals

		PPD in vivo	
		(+)	(-)
BCG in vitro	(+)	17	5
BCG in vitro	(-)	1	13

Lymphocytes from 36 individuals who had been recently vaccinated with BCG (3 months to 2 years), were boosted in vitro with BCG. The same individuals were also tested with PPD (see legend to Table 4). $P < 0.0001$ when the in vitro and in vivo responses were compared by Fisher's exact test. The gamma variable was calculated as 0.956

varied with the spontaneous level of NCMC (Table 3); thus, PBL from 63% of the persons with normal NK activity could be stimulated, while only 19% of those with low NK activity showed boosting of NK activity with BCG ($P < 0.001$). The same correlation was also observed when individuals were grouped by sex or by age (results not shown).

6. In vivo Relevance

We tested the reactivity of our healthy donors to PPD and correlated their in vivo response to PPD with their response to in vitro boosting with BCG. For this study only subjects that were known to have been previously vaccinated with BCG were selected. Table 4 shows that in the 128 healthy individuals tested, a strong correlation ($P < 0.004$, concordance = 0.541) exists between the number of persons whose NK activity could be boosted in vitro with BCG and those who responded positively to PPD in vivo. An even stronger correlation ($P < 0.0001$, concordance = 0.956) was demonstrated in the same control population when PPD tests were performed in individuals who were known to have been recently vaccinated (within 2 years) with BCG (Table 5).

Discussion

We provide direct evidence demonstrating that in a normal adult population the NCMC level is an individual trait that varies very little under physiological conditions, although a wide range of NK activity is observed overall in the population.

We found in our normal population that the range of NK activity varied from 4% to 46% lysis at the E/T ratio of 5/1. We defined as low NK activity a percent lysis of less than 15% and 19% of our normal population exhibited this low level of NK activity. We established that within this population, men have an overall statistically significantly higher level of NCMC activity than women ($P < 0.0004$). The lower NCMC level of women can be explained by the observation that 27% of women in our study, as against 8% of men ($P < 0.0001$), have little or very low levels of NK activity, i.e., they consistently fall below the 90% tolerance limit (less than 15% lysis). Although sex differences in NK activity have been previously reported for humans and rodents [15, 19, 21] no explanations for the difference have been suggested. Our results, on the other hand, suggest that the sex difference is due to a segment of the female population who consistently exhibit low NK activity.

How and why this depression of NK activity might occur is as yet unknown. Since this low NK activity is found

predominantly in young women we suspect that sex hormones may be involved. In support of our observations, in female mice the sustained administration of 17 β -estradiol [29] and hypophysectomy [28] suppress NK activity in vivo. In his study, Saxena [28] suggests that growth hormone may play an important role in the homeostasis of NK cells. Based on these observations we suspect a difference in the hormonal levels between women with low NK activity and those with normal NK activity. However, until now we have failed to reveal any differences between women with low NK activity versus those with normal levels of NK activity when data on hormonal status, contraceptive intake, hysterectomies and time in menstrual cycle have been analysed. Research to determine whether there is indeed a hormonal influence on NK activity is currently under way in our laboratory by the direct measurement of blood levels of estrogens, growth hormone, and thyroid hormones in both groups of young women.

The low NK activity certainly involves genetic factors. Petranyi et al. [20] have presented evidence that in man genetic factors can regulate NK activity; hyporeactivity in males was associated with the HLA-A3 and B7 haplotypes. This finding was later confirmed and extended by Trinchieri et al. [32] and Santoli et al. [26], who reported an association between increased reactivity and the HLA-B12 haplotype. A correlation between HLA and the level of NCMC was not sought in the present study, although we have noticed that members of the same family have almost identical levels of NCMC. It would therefore be interesting to HLA-type our population to see whether we can also find a correlation between HLA and NK activity, particularly within the low-NK activity group. A number of other possible explanations can be advanced for the low NK activity in some individuals. Suppressor factor(s) or NK suppressor cells could be present or autoantibodies directed against the person's own cell surface antigens could result in an antibody-mediated cytotoxicity of the NK cells.

In the past few years, it has become clear that NK activity can be substantially augmented in vivo above normal spontaneous levels [reviewed in 5, 15] by a number of agents, including viruses, interferon, interferon inducers and immunostimulants such as *Corynebacterium parvum* and BCG [2, 12, 14, 25, 34]. In vitro treatment of effector cells with interferon, interferon inducers and retinoids [3] can also augment NK activity. We demonstrate in this study that addition of BCG to the test system can induce a striking boosting of NCMC with rapid induction of high levels of cytotoxicity. Moreover, the initial or spontaneous level of NCMC has been the single most important factor in in vitro boosting with BCG, i.e., 63% of donors with normal NK activity, as against only 19% of donors with low NK activity, could be stimulated with BCG. In mice a similar observation has been made with interferon stimulation. Herberman et al. [6] reported that strains with a high resting level of natural killing responded better to interferon than strains with low baseline natural killing. The failure of BCG (in vitro) to stimulate NK activity in subjects with both low and high spontaneous levels of NK activity suggests a genetic control of boosting. A lack of boosting could perhaps involve a suppression mechanism.

It is of particular interest that the percentage of normal individuals who can be boosted with BCG varies according to their spontaneous level of NCMC, as it has been reported [8] that in some people BCG vaccination, even when repeated several times, fails to induce delayed hypersensitivity to tuberculin (PPD). Furthermore, Neveu et al. [17] have shown

by in vitro transfer experiments that a nonadherent cell population is responsible for this unresponsiveness to PPD.

Data presented in this study show that only 56% of the total population (108 of 191) could be boosted with BCG. These data confirm our earlier observation [11] that 51% of healthy individuals (14 of 27) react positively to 5 IU of PPD. The unstimulated level of NCMC was the only important variable among those studied in determining in vitro reactivity to BCG, and could well explain the low response of many individuals to PPD. In fact, our in vivo data show that a great majority of donors who fail to react in vitro to BCG do not respond to BCG vaccination and/or repeatedly show a negative response to PPD in vivo (Tables 4 and 5) even after several BCG vaccinations. Furthermore, we have already demonstrated [12] that only 29% of healthy donors with low NK activity could be stimulated with either bacterial (BCG, *C. parvum*, *B. abortus*) or viral (influenza and polio vaccines) adjuvants as well as interferon. Our studies demonstrate that within the normal population a selective group of individuals have a consistently low level of NK activity and that in a great majority of cases, these people fail to be stimulated by adjuvants. Sequential studies on this group of individuals might help to determine the underlying causes of this 'deficiency'.

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