CELLULAR IMMUNITY TO BCG VACCINE IN NEWBORN
Yenidoğanlarda BCG aşısına karşı hücresel bağışıklık

Duran Arslan[1], H Basri Üstünbaş[2], Türkan Patıroğlu[2], Mustafa Kendirci[3]

Summary: *We designed this study to investigate cellular immunity to BCG vaccine more accurately with antigen spesified lymphocyte stimulation test (LST). Twenty four newborn babies (study group) and their 20 mothers and 12 non-vaccinated healthy children (control group) were studied. Study group babies were tested for white blood cell count (WBC), absolute lymphocyte count (ALC) and LST with PPD before and 7 to12 weeks following BCG vaccination. Control group babies were evaluated with the same tests. LST with PPD were performed to the twenty mothers of study group babies. WBCs and ALCs of all babies were consistent with expected values for their ages. Stimulation indexes of study group increased significantly after vaccination (p<0.002). It was observed that a small proportion of tuberculin immunity was transferred to the newborn. We concluded that cellular immunity of newborn babies was sufficient and BCG vaccination induced cellular immunity well.*

Key Words: *BCG vaccine, Cellular immunity, Lymphocyte transformation*

Özet: *Bu çalışma BCG aşısı ile gelişen hücresel immüniteyi antijen spesifik lenfosit stimülasyon testi kullanarak daha sağlıklı bir şekilde göstermek amacıyla sağlıklı 30 yenidoğan bebek ile bunlardan 20'sinin annesinde ve BCG aşısı yapılmamış 12 çocukta yapıldı. Çalışma grubundaki 24 bebeğe BCG aşısı yapılmadan önce ve BCG aşısı yapıldıktan 7-12 hafta sonra beyaz küre ve mutlak lenfosit sayıları ve spesifik antijen ile (PPD) lenfosit stimulasyonu testi yapıldı. Çalışma grubundaki bebeklerden 20'sinin annesine de lenfosit stimulasyon testi yapıldı. Henüz BCG aşısı yapılmamış, yaşları 2-4 ay arasında değişen 12 bebekte kontrol grubu olarak aynı testler çalışıldı. BCG aşısı yapıldıktan sonra bebeklerin stimülasyon indekslerinde oldukça anlamlı bir yükselme oldu (p<0.002). Kontrol grubu bebeklerin ortalama stimulasyon indeksleri, çalışma grubu bebeklerin aşılama öncesi dönemi ile benzer bulundu. Sonuçta BCG aşısının bölgemizde aşılama ile ilgili diğer teknik konulara dikkat edilmesi kaydıyla iyi bir koruyuculuk sağlayabileceği kanaatına vardık.*

Anahtar Kelimeler: *BCG aşısı, Hücresel immünite, Lenfosit transformasyonu*

Cellular immunity to BCG vaccination has been usually evaluated with tuberculin reactivity (1-4). Tuberculin reactivity is a delayed type hypersensitivity reaction in which T lymphocytes have major contribution. T lymphocytes which play role in delayed type hypersensitivity are different from those in cellular immunity. Tuberculin reactivity reflects a previous meeting with microorganism (m.tuberculosis) without developing resistance. At the same time immune system in skin and some local factors may effect tuberculin reactivity towards the negative side (5). Because of the reasons mentioned above and our controversial clinical experiences we decided to investigate cellular immunity to BCG vaccination more accurately with antigen spesified lymphocyte stimulation test.

MATERIAL AND METHODS

This study was carried out in Erciyes University Hospital. We had consents of all parents before

Erciyes Üniversitesi Tıp Fakültesi KAYSERİ
Pediatri Y.Doç.Dr.[1], Prof.Dr.[2], Doç.Dr.[3]

Geliş tarihi: 12 Mart 1997

study. Study group was 24 newborn babies, (14 male, 10 female) and their 20 mothers. Control group was 12 non-vaccinated healthy children (7 male, 5 female). None of the children or mothers had any disease. Study group was tested for white blood cell (WBC) count, peripheral blood smear and differential as well as lymphocyte stimulation test (LST) with purified protein derivative (PPD) before and 7 to 12 weeks (average 9.3±1.5) following BCG vaccination. Control group children were evaluated with the same tests. LST with PPD were performed to the twenty mothers of newborn babies. Fifteen of 24 newborn babies were evaluated for lymphocyte subgroups (CD3,CD4,CD8,CD16 and CD19).

Study group children received BCG vaccination intradermally in a dose of 0.05 ml (50.000 alive germ) at 1-5 (average 2.3±1.1) days of the life. The tests were repeated after 7-12 weeks (average 9.3±1.5) of the vaccination. Age of control group during evaluation ranged between 2-4 months (average 2.6±0.6). WBC were counted in capillary blood samples drawn from finger or heel with Coulter Counter Autoanalyser (S880). Peripheral blood smear was stained with Wright's stain and evaluated at 100x magnification with light microscope. Lymphocyte ratio in smear was multiplied with WBC values and absolute lymphocyte count calculated.

Two ml of venous blood sample was drawn into the unpreserved heparine (H3125,Sigma) flushed steril syringe for LST. Furthermore four ml of venous blood sample was drawn into the preserved heparine (Liquemine,Roche) flushed syringe for lymphocyte subgroup determination. Tests were performed immediately.

Lymhocyte separation: Blood samples were diluted with equal amounts of phosphate buffer solution (PBS) and mononuclear cells separated by ficoll-hypaque (Histopaque, Sigma) centrifugation method (6). Ultimately mononuclear cells were suspended in complete medium in a concentration of 10^6 cells/ml for LST and in PBS with 5% fetal calf serum in a concentration of $5x10^6$ cells/ml for lymphocyte subpopulation (7).

Lymphocyte Stimulation Test: To determine the dose of antigen and incubation time several doses of antigen and different incubation periods were tried before commencing to the study. It was decided that four days of incubation period and 0.05 mg dose of antigen (PPD) were optimal.

Three tubes for stimulation and one for unstimulation were prepared; 0.8 ml complet medium, 0.05 mg (0.1 ml=5 TU) PPD (antigen) and 0.1 ml (10^5) cells were added to stimulation tubes; 0.9 ml complet medium and 0.1 ml (10^5) cells combined into the unstimulation tubes. Samples were incubated in a medium containing 5% CO_2 at 37^0C for four days. To mark the cells radioactively, 0.5 µCurie of Methyl-^{3}H-Thymidine (Amersham) was put into the tubes. After 16 hours of incubation, to cease the reaction 3- methyl thymidine (Sigma) was added and kept at +40°C for an hour. Radioactively marked cells were adsorbed on filter discs and dried. The discs were washed in turn with 5% TCA, ethyl alcohole and acetone mixture and acetone alone and dried. Dried discs were added in scintillation fluid which contains primary flour. Radioactivity was read as count per minute (cpm) in a beta counter (RACKBETA). For each case four stimulated and two unstimulated cpm values were recorded. Cpm results obtained from scintillation fluid and empty (without cell) discs represented basal values. Mean values for stimulated , unstimulated and basal cpms were calculated separately. Stimulation index (SI) was calculated as follows (8):

$$\text{Stimulation Index (SI)} = \frac{\text{cpm (stimulated) - cpm (basal)}}{\text{cpm (unstimulated) - cpm (basal)}}$$

Lymhocyte subpopulations were detected with indirect immunoflourescein method (Behring Monoclonal Antibody). For statistical analysis Student t test and paired t test were used; p values <0.05 were considered as statistically significant.

Table I. Physical features of study and control groups

	n	Age*	Sex (M/F)	Weight* (gr)
Study group-Prevaccination	24	2.3 ± 1.3 day	14M/10F	3272 ± 456
Study group-Postvaccination (a)	24	2.7 ± 0.5 mo	14M/10F	6223 ± 672
Control group (b)	12	2.6 ± 0.6 mo	7M/5F	6020 ± 682
*X ± SD				
Statistics				
a and b		t=0.822 ; p=0.3		t=1.017 ; p=0.4

Table II. WBC and absolute lymphocyte counts and lymphocyte ratio in peripheral blood smear of study and control groups

Groups	n	WBC Count* (in mm^3)	Abs.Lymp.Count* (in mm^3)	Lymph. Ratio* %
Study group-Prevaccination (a)	24	14587 ± 4373	4681 ± 2053	31.9 ± 14.8
Study group-Postvaccination (b)	24	11379 ± 4533	6440 ± 2578	58.1 ± 13.8
Control group (c)	12	13316 ± 5942	6019 ± 2574	49.7 ± 18.5
*X ± SD				
Statistics				
a and b		t=2.552, p<0.02	t= -2.869;p<0.009	t= -6.688;p<0.0001
a and c		t=0.728;p=0.471	t= -1.957; p=0.059	t= -3.107;p<0.004
b and c		t=1.089;p=0.284	t= 0.463;p=0.646	t= 1.588;p=0.122

Table III. Stimulation Indexes of Study Group, Control Group and Mothers

Groups	n	Stimulation Indexes X ± SD
Study group-Prevaccination (a)	24	0.28 ± 0.55
Study group-Postvaccination (b)	24	1.3 ± 1.87
Control group (c)	12	0.5 ± 0.71
Mothers (d)	20	1.3 ± 1.67
Statistics		
a and b		t= -3.547 ; p<0.002
a and c		t= -0.966 ; p=0.326
b and c		t= 1.427 ; p=0.162
a and d		t= -2.803 ; p<0.008
b and d		t= 0.007 ; p=0.994

Table IV. Lymphocyte Subgroups of Newborn Babies

Monoclonal antibody	Ratio (%) X ± SD
CD3	51.73 ± 3.01
CD4	31.80 ± 4.72
CD8	24.07 ± 2.74
CD19	25.67 ± 8.48
CD16	14.87 ± 3.50
CD4/CD8	1.34 ± 0.15

RESULTS

Physical features of study (prevaccination and postvaccination) and control groups were presented in Table I. The study group consisted of 14 male and 10 female babies whereas the control group consisted of 7 male and 5 female babies. Average ages of prevaccination, postvaccination and control groups were 2.3±1.3 (1-5) days, 2.7±0.5 (2-3.5) months and 2.6±0.6 (2-4) months respectively. Postvaccination study group and control group were similar for age (p=0.3), sex and body weight (p=0.4).

WBC counts, lymphocyte ratios in peripheral blood smear and absolute lymphocyte counts of prevaccination and postvaccination study groups and control groups were shown in Table II. Postvaccination study group and the control group were similar for each of the three parameters (p=0.284, p=0.122 and p=0.646 respectively). Prevaccination study group and postvaccination study group were different for each of the three parameters (p<0.02, p<0.0001 and p<0.009 respectively). These results were consistent with expected values for their ages.

Stimulation indexes of three groups and mothers were presented in Table III. Stimulation indexes scattered in a wide range. Stimulation indexes of 22 (%91.7) babies increased after vaccination. Average stimulation index of postvaccination study group was higher than the prevaccination study group (p<0.002). Prevaccination study group and control group were similar for stimulation indexes (p=0.326). Postvaccination study group was similar to the control group (p=0.162) .

Average stimulation indexes of mothers were higher than the prevaccination study group ($p<0.008$) and similar to postvaccination study group ($p=0.994$). Lymphocyte subpopulations and CD4/CD8 ratios of 15 prevaccination study group babies were shown in Table IV.

DISCUSSION

Immune incompetency of newborn babies has been reported several times in the literature (9-11). There were also several reports regarding immune competency of newborn babies (12,13). In respect to immune competency of newborn Ildırım et al (2) reported that BCG vaccine administered in third month of life produced better immunity than that in the first month. Ormerod and Garnett (3) observed that tuberculin reactivity was still maintained after four years when vaccination was performed in the newborn period. In both studies immunity was determined with tuberculin reactivity. Because there are controversial results concerning immunity of the newborn, we studied WBC counts and absolute lymphocyte counts in all babies and lymphocyte subpopulations in some. WBC and absolute lymhocyte counts in all groups (prevaccination, postvaccination study groups and control group) were compatible with reference values of their ages (14). The values of CD8, CD16 and CD19 of 15 babies were within reference values. CD3 values were slightly lower than the normal range. CD4 values were within lower limits of the normal (15). Slightly low CD3 and CD4 values were attributed to study tecnique that was used in laboratory. In addition to lymphocyte subgroup values CD4/CD8 ratio is also important for immune competency and values of less than 1.2 were accepted as "low". CD4/CD8 ratios of all 15 babies were equal to or higher than 1.2 and we concluded that none of these newborn babies had cellular immune incompetence.

To date cellular immunity to BCG vaccination is usually evaluated by tuberculin reactivity. The relationship between tuberculin hypersensitivity and protective immunity has been researched in several studies (16-18). There are some evidences about tuberculin sensitivity stating that it is not always parallel to protective immunity. These are ; (i) no direct relationship has been found out between the size of skin test reaction and the degree of acquired immunity, (ii) some vaccinated animals with negative skin test were immune to the disease, (iii) tuberculin testing boosted a waning tuberculin reaction but did not boost a waning protective response and (iv) differences in allergenicity of a panel of BCG vaccines were more pronounced than differences in protective ability (19). Lymphocyte stimulation test evaluates cellular immunity. When it is carried out with a certain antigen it refers specified cellular immunity. We evaluated specified cellular immunity to BCG vaccine by the help of lymphocyte stimulation test. We used PPD as the antigen. Median stimulation indexes of postvaccination study group were higher than those in the prevaccination group ($p<0.002$). There was a strong correlation between pre and post vaccination stimulation indexes (coefficient of correlation=0.863, $p<0.0001$). Stimulation indexes were increased in 91.7% and decreased in 8.3% of the study group babies after vaccination. We suggested that some babies were not vaccinated properly. When children were evaluated individually stimulation indexes increased by 32-fold (average) after vaccination. Ten fold or more increase in stimulation index may be accepted as sufficient (20). Since there are no reference values about LST, we can not comment on protective value of this increase in stimulation index. Stimulation indexes after vaccination were similar with healthy mothers ($p=0.768$).

Tubercle bacilli has many antigenic determinants. Some of them are specific to m.tuberculosis but others are not (21). The microorganisms that are antigenically similar to tubercle bacilli may effect immunity to BCG vaccination positively or negatively (22, 23). A good example of these organisms is atypical mycobacteria. We evaluated the effect of atypical mycobacteria on nonvaccinated control group. Stimulation indexes of the control group were similar with prevaccination study group

(p=0.842), and were lower than postvaccination control group ($p<0.05$). We concluded that atypical mycobacteriae constitute a low grade sensitivity and it has a positive effect on BCG vaccine immunity.

Previous investigators suggested that tuberculin immunity may be transferred through human breast milk (24,25). Some others found out evidence for transplacental transfer of tuberculin immunity (7,24,26). We performed lymphocyte stimulation test in 20 mothers of study group babies to evaluate the "mother-to-infant" transfer of cell mediated immunity to tubercle bacilli. Average stimulation indexes of mothers were higher than their newborn babies ($p<0.008$). There was no correlation between babies and mothers. In previous studies it was found that PPD induced blastogenesis was depressed in the last four weeks of pregnancy and at the time of delivery. This response increased in time and in 4 to 6 weeks of life reached peak levels then waned and disappeared in the third month of life (7,24). Since we have vaccinated our babies in the newborn period we were unable to evaluate them in 4th to 6th weeks of life.

Finally we concluded that (i) cellular immunity of newborn is sufficient and we can vaccinate them with BCG , (ii) in newborn BCG vaccination induce cellular immunity well, but whether cellular response is high enough to protect them against tuberculosis remains unclear; this may be the subject of another study, (iii) atypical mycobacteriae in our region may effect immunity to BCG vaccine positively, and (iv) a small proportion of tuberculin immunity is tranferred to the newborn.

REFERENCES

1. *Clarke A, Rudd P. Neonatal BCG immunization. Arch Dis Child 1992; 67: 530 - 531.*
2. *Ildırım İ, Sağan N, Çavuşoğlu B. Comparison of BCG vaccination at birth and at third month of life. Arch Dis Child 1992; 67:80-82.*
3. *Ormerod LP, Garnett JM. Tuberculin skin reactivity four years after neonatal BCG vaccination. Arch Dis Child 1992; 67:530-531.*
4. *Şahin N, Fazlı ŞA. Yenidoğanlarda BCG aşısının koruyuculuğu. XI. Gevher Nesibe Tıp Günleri Özet Kitabı, Kayseri 1993, ss 61 .*
5. *Piessens WF. Introduction to the immunology of tuberculosis. Rev Infect Dis 1989; 11:436-442.*
6. *Boyum A. Separation of leucocytes from blood and bone marrow. Scand J Lab Invest 1978; 21:77.*
7. *Keller MA, Rodriguez AL, Alvarez S, et al. Transfer of tuberculin immunity from mother to infant. Ped Res 1987; 22: 277-281.*
8. *Stites P. Clinical Laboratory Methods for Detection of Cellular Immunity. In: Stites P, Terr Al (eds). Basic and Clinical Immunology, Appleton and Lange, Lebonan 1991, pp263-283.*
9. *Wilson CB. Immunologic basis of increased susceptibility of neonate to infection. J Pediatr 1986; 108:1- 12.*
10. *Winter HS, Gard SE, Füscher TJ, et al. Deficient lymphokine production of newborn lymphocytes. Pediatr Res 1983; 17: 573 - 578.*
11. *Taylor S, Bryson YJ: Impaired production of g-interferon by newborn cells in vitro due to a functionally immature macrophage. J Immunol 1985; 134: 1493-1497.*
12. *Handel ZT, Levin S, Dolphin Z, et al. Immune competence of newborn lymphocytes . Pediatrics 1980; 65: 491- 496.*
13. *Hicks MJ, Jones JF, Thies AC, et al. Age - related changes in mitogen - induced lymphocyte function from birth to old age. Am J Clin Pathol 1983; 80: 159-163.*
14. *Nathan DG, Oski A. Reference values in infancy and childhood. In: Hematology of infancy and childhood. WB Saunders Company 1987, pp 1677-1700.*
15. *Lanier L. Cells of immune response: Lymphocytes and mononuclear phagocytes. In: Stites P, Terr AI (eds). Basic and Clinical Immunology, Appleton and Lange, Lebonan 1991, pp 61-72.*

16. Jespersen A, Weis-Bentzon M. Relationship between tuberculin sensitivity and acquired resistance in guinea-pigs vaccinated with BCG strains of different virulence. Acta Pathol Microbiol Scand 1967; 71:114-131.
17. Tolderlund K, Bunc-Christensen K, Guld J. Duration of allergy and immunity in BCG vaccinated guinea-pigs. A five years study. Bull WHO 1967;36:759-769.
18. Tuberculosis Program Public Health Service, USA: Experimental studies of vaccination allergy and immunity in tuberculosis V. Relation between degree of tuberculin sensitivity and resistance in the vaccinated individuals. Am J Hyg 1955; 62:185-189.
19. Smith DW. Protective effect of BCG in experimental tuberculosis. Adv Tuberc Res 1985; 22:1- 99.
20. Oppenheim JJ, Dougherty S, Chan SP, et al. Use of lymphocyte transformation to assess clinical disorders. In: Uyas GN, Stites D, Brecher G (eds). Laboratory diagnosis of Immunological Disorders. Grune and Stratton, 1985, pp 87-109.
21. Fine PEM. The BCG story: Lessons from past and implication for future. Rev Infect Dis 1989; 11: 353- 359.
22. Kulkarni S, Kamat RS. Cross-reactions in cell mediated immunity induced by atipical mycobacteria.J Med Microbiol 1986 ;21:35-38.
23. ten Dam HG: Research on BCG vaccination. Adv Tuberc Res 1984 ;21: 79 -106.
24. Schlessinger JJ, Covelli HP. Evidence for transmission of lymphocyte responces to tuberculin by breast feeding. Lancet 1977; 2: 529 - 532.
25. Mohr JA. The possible induction and/or acquisition of cellular hypersensitivity associated with ingestion of colostrum. J Pediatr 1973; 82:1062-1064.
26. Pabst H, Crawford J, Grant M, Boyce S. Transfer of cell-mediated immunity (CMI) to the fetus. Ped Res 1984; 18: 262.