Lymphoproliferative response of Fanconi anemia patients to mitogens, bacterial and viral antigens in vitro

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Summary
Increased susceptibility to infections in Fanconi anemia (FA) have been investigated by determining immune response capability of the patients against to mitogenic, bacterial and viral antigens *in vitro*. Peripheral blood mononuclear cells (PBMC) were obtained from 11 FA patients and 11 healthy control individuals. 10^5 cells from each sample were assessed by using colorimetric MTT assay including 20 µg/ml pytoheamagglutinine (PHA), 10 µg/ml pokeweed mitogen (PWM), 6 µg/ml Tetanus toxoid (TT), 10 µg/ml purified protein derivative of mycobacterium (PPD) and 1.25 µg/ml cytomegalovirus (CMV) antigen. Lymphoproliferative response to PHA, PWM, TT and PPD were found significantly lower in FA patients than control group. Lymphoproliferation to CMV antigen was significantly higher in CMV seropositive than seronegative FA patients. To conclude, the lower lymphoproliferative response capability to mitogens and bacterial antigens in FA patients implied susceptibility to infections, but, it is likely in CMV seropositive FA patients, the cells encountering antigens could become immunopotent.
Key words
Fanconi anemia, lymphoproliferation, mitogen, cytomegalovirus

Fankoni anemili hastaların mitojenlere, bakteriyel ve viral antijenlere karşı *in vitro* lenfoproliferatif yanıtları
Özet
Fankoni anemili hastaların enfeksiyonlara karşı artmış hassasiyeti, hastaların mitojenlere, bakteriyel ve viral antijenlere karşı immün cevap yetenekleri *in vitro* ortamda belirlenmek suretiyle incelenmiştir. Periferik kan mononükleer hücreleri (PBMC) 11 Fankoni anemili hasta ve 11 sağlıklı kontrol bireyinden alınmıştır. Her bir örnekten 10^5 hücre alınarak MTT yöntemiyle, 20 µg/ml fitohemaglutinin, (PHA), 10 µg/ml pokeweed mitojen (PWM), 6 µg/ml Tetanoz toksoidi (TT), 10 µg/ml saflaştırılmış mycobacterium proteini (PPD) and 1.25 µg/ml citomegalovirus (CMV) antijeni ile test edilmiştir. PHA, PWM, TT ve PPD' ye karşı lenfoproliferatif yanıt kontrol grubuna göre Fankoni anemili hastalarda anlamlı şekilde düşük bulunmuştur. CMV seropozitif hastalarda ise CMV antijenine karşı lenfoproliferatif yanıt CMV seronegatif hastalardan daha yüksek bulunmuştur. Sonuç olarak, Fankoni anemili hastalarda mitojenlere, bakteriyel ve viral antijenlere karşı düşük lenfoproliferatif yanıtın infeksiyonlara yatkınlığın bir işareti olduğunu fakat CMV seropozitif hastalarda görüldüğü gibi antijenle temas durumunda hücrelerin immunopotent hale dönüşebildiğini söyleyebiliriz.
Anahtar Kelimeler
Fanconi anemisi, lenfoproliferasyon, mitojen, citomegalovirus

Introduction

Fanconi Anemia (FA) is an x-linked heterogeneous autosomal recessive genetic disorder characterized by congenital abnormalities, progressive bone marrow failure, and cancer susceptibility (1, 2). Defective hematopoiesis, defective DNA repair and altered levels of certain growth factors (3, 4) in FA may affect hematopoiesis by altering the bone marrow microenvironment, leading to dysregulation of cellular homeostasis, differentiation and response to stress. (5-9)

Patients with FA frequently suffer from bleeding and infection, symptoms related to thrombocytopenia and neutropenia (10). FA may include increased susceptibility to oral diseases with underlying host defense impairment coupled with periodontal infection by cytomegalovirus (CMV) and *Actinobacillus actinomycetemcomitans* (11, 12). A V*ibrio cholera* sepsis was reported in a child with FA which was likely to be caused by the underlying hematological complications (13).

The aim of this study was to investigate the susceptibility to infections of FA patients by determining lymphoproliferative response of the peripheral blood mononuclear cells (PBMC) to specific and nonspecific stimulants *in vitro*.

Patients and Methods

Blood samples were obtained from 11 FA patients (mean age 9.3 years; range 6 – 17) and 11 healthy individuals (mean age 8.9 years; range 3 – 13). The diagnoses of patients were confirmed by nitrogen mustard and/or diepoxybutone method to induce chromosomal

breakages in peripheral blood lymphocyte cultures (14). FA patients and control group individuals had primary immunization with tetanus toxoid (TT), Bacillus Calmette Guerin (BCG), diphtheria toxoid (DT), pneumococcus (Pnc), *Haemophilus influenzae* type b (Hib) and other childhood vaccines.

Lymphocyte stimulation assay

PBMC were obtained from heparinized venous blood over a Histopaque gradient (Sigma) according to the manufacturer's instructions. The lymphocyte band (at the interface) was carefully removed and washed in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) fetal calf serum, 1% L-glutamine (2.0 mM), 100 UI/ml penicillin and 100 µg/ml of streptomycin (complete medium).

The assay was performed in tissue culture plates (96 wells); the wells were fulfilled with complete medium followed by 20 µg/ml phytohemagglutinin (PHA, Biochrom KG, Berlin, Germany), 10 µg/ml pokeweed mitogen (PWM, Sigma Chemical Co., St. Louis, MO, USA), 6 µg/ml tetanus toxoid (TT, Aventis Pasteur SA, Lyon, France), 10 µg/ml purified protein derivative of mycobacterium (PPD, B-NCIPD Ltd. Sofia, Bulgaria), 1.25 µg/ml cytomegalovirus antigen (CMV, Abbott Lab. Abbott Park, IL) and PBMC. Culture plates were incubated at 5% CO_2 and 37 ^{o}C for 72 hours prior to MTT assay.

Cell proliferation was quantified by a colorimetric assay based on the reduction of [3-(4,5-dimethylthyazol-2yl)-2,5-diphenyl tetrazolium] (MTT). MTT solution (5 mg/ml in RPMI 1640 medium) was added and incubated (4 hours) at the same condition. Plates were centrifuged, supernatant was removed and 100 µl of dimethyl sulphoxide, DMSO (Sigma), was added under agitation; plates were read on an ELISA reader at 540 nm using 620 nm as reference filter (Organon Technica Reader Model 530).

CMV serology of FA patients

CMV seropositivity for specific IgM antibodies in sera was determined with CMV IgM microparticle enzyme immunoassay (MEIA) (15) by Abbott Axsym® system (Abbott Laboratories, Diagnostics Division, Abbott Park IL).

Statistical analyses

Results are expressed as mean and ± standard error of the mean (SEM). Significances between FA and control groups were determined by ANOVA.

Results

PHA and PWM mitogenesis

In order to assess T cell function, PBMC were cultured in the presence of PHA (20 µg/ml). Cultures were assessed for proliferation by the MTT assay as shown in Figure 1. PBMC obtained from FA patients exhibited lower proliferative responses to PHA than healthy individuals. The average absorbance for control group and FA patient PBMC were 0.243 (SEM=0.008) and 0.176 (SEM=0.006), respectively, ($p<0.05$).

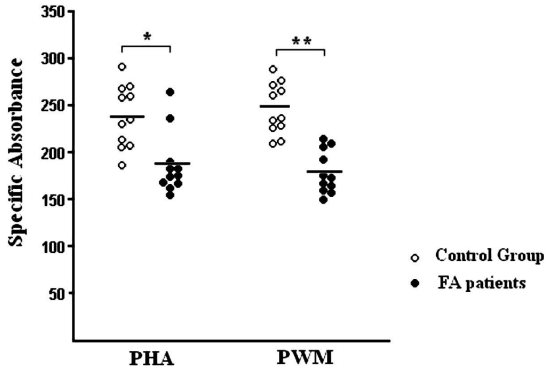

Figure 1

Mitogen induced lymphoproliferation of healthy control individuals and FA patients. PBMC cultured in triplicate in the presence of PHA (20 µg/ml), PWM (10 µg/ml). Following 72 h of culture, proliferation was assessed by MTT reduction. Each points represent mean specific absorbance (absorbance 540 nm with 620 nm Reference filter) for PHA and PWM treated cultures. *$p<0.05$, **$p<0.001$

In order to assess both T and B lymphocyte function, PBMC were cultured in the presence of PWM (10 µg/ml). Cultures were assessed for proliferation by the MTT assay as shown in Figure 1. PBMC obtained from FA patients exhibited lower proliferation response to PWM, compared to healthy control individuals. The average absorbance for control group and FA patient PBMC were 0.236 (SEM=0.009) and 0.185 (SEM=0.010), respectively, ($p<0.001$).

Lymphocyte response to bacterial antigens

To further assess lymphocyte function, PBMC were cultured in the presence of TT (6 µg/ml) and PPD (10 µg/ml). Cultures were assessed for proliferation by the MTT assay as shown in Figure 2. PBMC obtained from FA patients exhibited lower proliferation responses to TT and PPD, compared to healthy control individuals. The average absorbance of control group and FA patients' PBMC for TT were 0.254 (SEM=0.010) and 0.187 (SEM=0.007), respectively, ($p<0.001$). The average absorbance of control group and FA patients' PBMC for PPD were 0.241 (SEM=0.009) and 0.160 (SEM=0.012), respectively, ($p<0.001$).

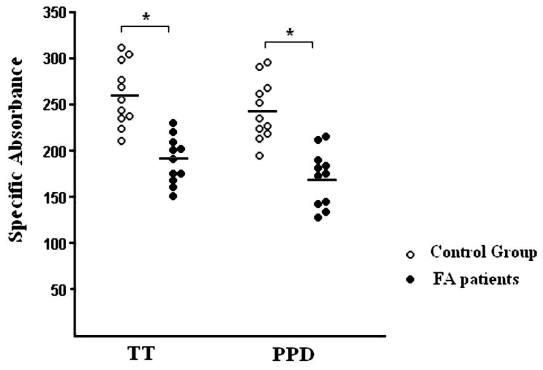

Figure 2
Bacterial antigen induced lymphoproliferation of healthy control individuals and FA patients. PBMC cultured in triplicate in the presence of TT (6 µg/ml), PPD (10 µg/ml). Following 72 h of culture, proliferation was assessed by MTT reduction. Each points represent mean specific absorbance (absorbance 540 nm with 620 nm Reference filter) for TT and PPD treated cultures. *p<0.001

Lymphocyte response to CMV

FA patients PBMC were tested for CMV infection. In 5 of 11 patients was detected anti-CMV IgM (+). PBMC form healthy control individuals and CMV seronegative and seropositive FA patients were cultured in the presence of CMV (1.25 µg/ml). Cultures were assessed for proliferation by the MTT assay as shown in Figure 3. The average absorbance of control group, CMV seronegative and seropositive were 0.254 (SEM=0.009), 0.184 (SEM=0.003) and 0.242 (SEM=0.013), respectively. Thus, p was less than 0.001 between controls and seronegatives, but less than only 0.05 between the formers and seropositives.

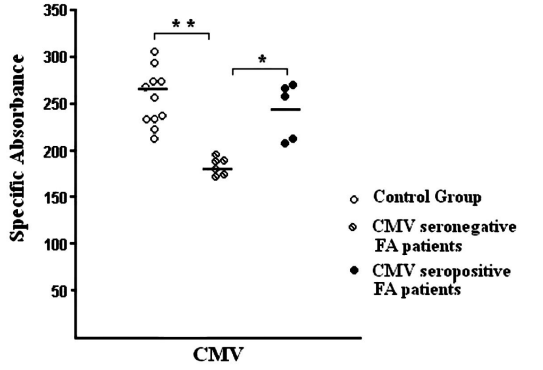

Figure 3
CMV induced lymphoproliferation of healthy control individuals, CMV seronegative and CMV seropositive FA patients. PBMC cultured in triplicate in the presence of CMV (1.25 µg/ml). Following 72 h of culture, proliferation was assessed by MTT reduction. Each points represent mean specific absorbance (absorbance 540 nm with 620 nm Reference filter) for CMV treated cultures. *p<0.05, **p<0.001.

Discussion

The present study suggests the immune status of FA patients under stimulation with non-microbial and microbial agents. PHA and PWM are potent, but non specific stimulants of lymphocytes. PHA is an effective stimulator of T lymphocytes, whereas PWM stimulates T, B lymphocytes and natural killer (NK) cells. PWM induces also proliferation and differentiation of B lymphocytes (16-18). After incubation of 72 hours with PHA and PWM, FA patients' PBMC had lower lymphocyte response to PHA and PWM, compared to the control group (Figure 1). The lower lymphoproliferative responses to the mitogens imply that immune system major cells have a general immune defect in FA patients.

TT is a toxin releasing from *Clostridium tetani* which promotes IgG producing B lymphocytes (19). PPD is a mycobacterium derivate protein reacting with PPD-reactive T lymphocytes (20). FA patients' PBMC in response to stimulation of TT and PPD were found decreased compared to control group (Figure 2). This showed that FA patients' PBMC have lower activation and proliferation capabilities by stimulation of these bacterial antigens. FA patients and control group individuals had primary TT and PPD vaccination. FA patients could not produce adequate immune response against these antigens, but control group had higher lymphocyte response. This suggest that FA patients have immune defects which may cause susceptibility to infections.

CMV is an opportunistic microorganism and affects immune defective or immunocompromised patients (21). Although CMV IgM antibodies have no importance in determining acute infections, a reactivation of the virus can be detected even in ended acute infection (22, 23). To discriminate CMV infected patients from non infected ones, we investigated CMV IgM antibodies in sera. CMV seropositive FA patients and control group had similar level lymphocyte proliferation, but CMV seronegative FA patients had significantly lower level than both former groups (Figure 3). These findings suggest that antigen encountering causes augmentation of immune response capability in CMV seropositive FA patients.

To conclude, susceptibility of FA patients to infections may be due to low immune response capability, but the immune system of these

patients is not completely insensitive to external stimulants as shown at CMV seropositive FA patients with high level lymphocyte response against CMV. Future studies are needed to explain high level lymphocyte responses against CMV in seropositive FA patients.

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