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Impaired immune function in children with Fanconi anaemia

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Summary

Fanconi anaemia is an autosomal recessive or X-linked disease characterized by progressive bone marrow failure, variable congenital abnormalities and a predisposition to malignancy. Reports of immune function in this population are limited, and include only specific areas of immune performance, showing variable defects. We report a cross-sectional immunological assessment in 10 children with FA. Absolute numbers of B cells and natural killer (NK) cells were reduced compared to controls ($P = 0{\cdot}048$ and $P = 0{\cdot}0002$, respectively), while absolute number of T cells were within normal range. Perforin and granzyme content of NK cells was reduced ($P < 0{\cdot}00001$ and $P = 0{\cdot}0057$, respectively) along with the NK cell cytotoxicity ($P < 0{\cdot}001$). Antigen proliferation in response to tetanus was decreased ($P = 0{\cdot}008$) while responses to candida and phytohaemagglutinin were not. Cytotoxic T cell function was also reduced ($P < 0{\cdot}0001$). Immunoglobulin G levels were normal in those evaluated. Our series represents the first attempt at a comprehensive quantitative and functional evaluation of immune function in this rare group of patients and demonstrates a significant deficit in the NK cell compartment, a novel quantitative B cell defect, along with abnormal cytotoxic function. These findings may be especially relevant in this patient population with known predisposition to DNA damage and malignancy.

Keywords

Fanconi anaemia; children; bone marrow failure; immunology

Fanconi anaemia (FA) is a rare inherited autosomal recessive or X-linked disease of increased sensitivity to DNA damage characterized by progressive bone marrow failure,

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variable congenital abnormalities and a predisposition to malignancy, particularly acute myeloid leukaemia (AML) and gynaecological and head and neck squamous cell carcinomas (HNSCC) (Auerbach & Allen, 1991; Alter, 2003). FA is associated with homozygous or biallelic mutations in one or more of the 14 known genes that play a role in the biochemical pathway of DNA repair, resulting in aberrant repair of double-strand DNA breaks (Taniguchi & D'Andrea, 2006; Wang, 2007). Clinically, there has been indication that some children with FA have an increased frequency of infection in general, not completely explained by neutropenia. The risk of HNSCC is 1000-fold greater in patients with FA than that of the general population, and also occurs at an earlier ags. HNSCC occur even after successful bone marrow transplantation and are associated with dismal prognosis. Chemotherapy and radiation therapy are associated with high morbidity and mortality, related to their underlying hypersensitivity to DNA damage(Masserot *et al*, 2008).

The human immune system recognizes, eliminates, and protects the body from viral and bacterial infections, as well as from transformed cells (pre-cancer cell) (Masserot *et al*, 2008). A variety of immune cells are actively involved in this process of immune surveillance, including B and T-lymphocytes, NK-cells, dendritic cells (DC), macrophages, and polymorphonuclear leucocytes (Whiteside *et al*, 1986). HNSCCs and anogenital SCCs [often human papillomavirus (HPV)-associated in the general population], are the most common solid tumours in patients with FA and generally carry a dismal prognosis (Kutler *et al*, 2003a). There are conflicting reports in the literature regarding the prevalence of HPV in FA-related HNSCCs. In a study of 25 patients with FA and HNSCCs, Kutler *et al* (2003b) demonstrated a much higher HPV prevalence in FA-related tumours (84%) compared to the general population (36%), this was not, however, replicated in a separate smaller study (van Zeeburg *et al*, 2008). Humoral immunity to the viral capsid has been shown to be sufficient for protection against infection, while innate and adaptive cell-mediated immunity appears important for the eventual elimination of HPV infection. However, molecular and cellular mechanisms responsible for the protection from and clearance of HPV infection are not completely established (Bleesing, 2004). With improvement in haematopoietic cell transplantation (HCT) outcomes of patients with FA, an increasing number of patients are surviving into adulthood and remain at risk for later malignancies (Kutler *et al*, 2003a). Thus, attention to immune functions in these patients is timely and critical to the assessment of likelihood of responsiveness to cancer vaccines for these high risk patients (Ressing *et al*, 2000; Frazer *et al*, 2004).

While a few studies to date have reported poorer immunological function in patients with FA and their families (Castello *et al*, 1998; Suzergoz *et al*, 2008), these studies have been quite small and limited. Along with lymphocyte dysfunction (Froom *et al*, 1987; Petridou & Barrett, 1990; Lebbe *et al*, 1993), high levels of tumour necrosis factor alpha (TNF-α) (Schultz & Shahidi, 1993; Rosselli *et al*, 1994), and low production of interleukins (IL) including IL1, IL2, and IL6, as well as interferon gamma (IFN-γ) and granulocyte-macrophage colony stimulating factor (GM-CSF) have also been reported (Roxo *et al*, 2001). In this study we performed a systematic assessment of adaptive immunity in 10 children with FA. Decreased levels of B and NK cells and cytotoxic function of NK cells and T cells show significant but heterogeneous immune deficiencies in these children.

Materials and methods

Subjects

The study included 10 patients with Fanconi anaemia, confirmed by diepoxybutane testing and, in the majority of cases, complementation group testing (Table I). The protocol was approved by the Cincinnati Children's Hospital Medical Center's (CCHMC) Institutional Review Board.

Immunological assessment

Assays were performed at the Diagnostic Immunology Laboratories at CCHMC. Results were interpreted with respect to age-appropriate reference ranges established in the laboratory. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by centrifugation with lymphocyte separation media (MP Biomedicals, Solon, OH, USA). Evaluation of patient lymphocyte subsets was performed via routine four-colour flow cytometric analysis of EDTA-preserved whole blood using fluorochrome-labelled monoclonal antibodies to lineage-specific cell surface markers for T cells (CD3, CD4, CD8), B cells (CD19), and natural killer (NK) cells (CD16, CD56) (Bleesing, 2004). All antibodies were obtained from BD Biosciences (San Jose, CA, USA). Briefly, erythrocytes were lysed by incubation in FACSLyse (BD Biosciences). Samples were then stained with antibody and analyzed on a FACSCalibur flow cytometer (BD Biosciences) using multiset software (BD Biosciences). Live cells were isolated based on forward- and side-scatter properties and gated to dot-plots to identify T cells (either CD3+CD4+ or CD3+CD8+), B cells (CD3−CD19+), and NK cells (CD3−CD16+CD56+).

Measurement of intracellular perforin and granzyme B was performed as previously described (Kogawa *et al*, 2002; Molleran Lee *et al*, 2004). Briefly, whole blood samples were surface stained with the following antibodies: CD8-peridinin chlorophyll protein (CD8-PerCP), fluorescein isothiocyanate-labelled T cell receptor (TCR)-αβ (BD Biosciences), and CD56-allophycocyanin (CD56-APC) (Beckman Coulter, Brea, CA, USA). Red cells were then lysed with FACSLyse (BD Biosciences) and washed. The white cell pellets were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences) and stained with either phycoerythrin (PE)-conjugated mouse IgG1 anti-granzyme B or PE-conjugated mouse IgG2b antiperforin (BD Biosciences) antibodies. After being washed, cells were resuspended in 1% paraformaldehyde and flow cytometry was performed using a FACSCalibur instrument. facsdiva software (BD Biosciences) was used to analyze granzyme B- or perforin-stained cells using histograms gating the NK cell population (TCR-*αβ*-negative and CD56-positive) and restricted to a live cell gate based on forward *versus* side scatter properties. The perforin-positive or granzyme B-positive regions were set by comparison to samples stained with PE-labelled Isotype control antibody, and the percentage positive for each gate was reported.

Cytolytic effector function for PBMC-derived NK cells and cytotoxic T lymphocytes (CTLs) was assessed using standard chromium-51 (^{51}Cr) release assays as previously described (Egeler *et al*, 1996; Molleran Lee *et al*, 2004). Briefly, for NK functional assessment, K562 target cells were labelled with ^{51}Cr and plated with effector cells from

patient-derived PBMC at a range of concentration ratios (typically 50:1, 25:1, 12·5:1, and 6·25:1). K562 (ATCC) is a bone marrow-derived human chronic myeloid leukaemia cell line. K562 cells were cultured in 20% fetal bovine serum, 5% penicillin/streptomycin and 5% L-glutamine for 7 d at 37°C in a 5% CO_2 atmosphere. For CD8+ CTL assessment an Epstein-Barr virus (EBV)-immortalized donor PBMC-derived cell line (AS) was utilized as target cells. Patient-derived PBMC were incubated with irradiated target AS cells for 7 d, for sensitization, then plated with ^{51}Cr labelled target AS cells as described above. Co-cultures were then incubated for 4 h at 37°C and 5% CO_2, and supernatants were assessed by gamma scintillation for ^{51}Cr release. The percentage cytotoxicity for each effector: target (E:T) ratio was determined according to the following formula: % cytotoxicity = 100 × (mean experimental sample radioactivity−mean background radioactivity) / (mean maximum radioactivity−mean background radioactivity). Plots were generated with 'E:T ratio' on the *X*-axis and 'percent lysis' on the *Y*-axis, with one 'lytic unit' defined as the number of effector cells necessary to give a certain level of cytotoxicity (10% for NK cells, 25% for CTLs),determined from the cytotoxic dose-response curve. T cell proliferation was measured by [^{3}H] thymidine incorporation into PBMCs following incubation with optimal concentrations of the mitogen phytohaemagglutinin (PHA) (Egeler *et al*, 1996), or candida or tetanus-derived antigens (Monafo *et al*, 1992). Briefly, patient-derived PBMCs were plated with appropriate concentrations of PHA, candida or tetanus-derived antigens and incubated for 72 h (PHA) or for 6 d (Candida/Tetanus) at 37°C and 5% CO_2. Cultures were then pulsed with [^{3}H] thymidine and incubated for an additional 12–24 h, after which cells were harvested using a Packard Cell Harvester and assessed for [^{3}H] thymidine incorporation by scintillation using a TopCount NXT. Immunoglobulin G levels were determined by standard methods in the CCHMC clinical laboratory.

Statistical analysis

Ten patients with FA were assayed and compared with population references for CD19, CD16, CD3, NK cell function, response to different mitogens, CTL function, perforin (% of NK-cells) and granzyme B (% of NK-cells). Age-specific references were used to account for the immune function changes in response to growth and development.

Derived variables

Outcomes with available clinical standards (NK cell function, Tetanus, Candida, CTL function, and PHA) were dichotomized into normal and abnormal groups according to the age-specific standards. For outcomes with available population mean and standard deviation (SD) (NKperforin and NKgranzyme B), the age-specific *z*-score was calculated (observation value−mean/SD). For outcomes with population median and fifth and 95th percentiles available, *z*-score was calculated as previously described (Liou *et al*, 2001). Quantitative values were standardized for age by *z*-score to allow evaluation within the context of each patient's normal range due to the variance of normal range with age.

Briefly, the upper and lower standard deviation for each age group was calculated as following:

$$\text{Upper SD} = (95\text{th percentile} - \text{median})/1{\cdot}64$$

Lower SD = (median − 5th percentile)/1·64

For values above the median, *z* score = (observed value−median)/upper SD; for values below median, *z* score = (observed value−median)/lower SD.

Statistical test

One-sided one sample *z*-test was used for immune functions with *z*-score. We tested the hypothesis of whether our FA patients had lower *z*-scores than the reference sample (mean = 0, SD = 1). For dichotomized outcomes, one-sided binomial tests were used. We tested the hypothesis of whether the proportion of FA patients with normal measurements was lower than 97·5%. Since the clinical standards used were set as mean−2 SD of the reference population, 97·5% of the individuals from the reference population were expected to have normal measurements. sas 9.2 software was used for data analyzes and uncorrected p-values are reported, without Bonferroni correction.

Results

Patient characteristics and haematological findings at the time of study are shown in Table I. Femailes accounted for eight of the ten patients, and all but two were of the most common complementation group, Group A. The majority of patients, eight of ten, had clinically stable haematological disease status at the time of evaluation. None of the patients were receiving supportive therapy with granulocyte colony-stimulating factor. Patient 7 had severe marrow failure, had been receiving androgen therapy (fluoxymesterone) for 16 months before it was discontinued 1 month prior to evaluation, and was undergoing evaluation for bone marrow transplantation. Patient 4 also had severe marrow failure and was close to evaluation for bone marrow transplantation.

Quantitative and qualitative analysis of lymphocyte subsets, including T cells, B cells and NK cells, are shown in Table II. Overall, only one of ten children evaluated was normal in all parameters tested. Five of 10 children had low B cell numbers (absolute CD19) and only one of 10 had low T cell numbers (absolute CD3). Two of 10 children had low NK cell numbers (absolute CD16/56), however, all eight of the remaining children had absolute NK cell numbers in the lower third of the normal range for age. These quantitative measures were compared to normal controls by conversion to age-specific *z* scores in order to account for changes in normal distribution with age. The mean *z* scores of absolute numbers of CD19 positive B and CD16/56 positive NK cells were lower compared to controls ($P = 0{\cdot}048$ and $P = 0{\cdot}0002$), while no difference in the *z* score of the absolute numbers of CD3 positive T cells was detected between patients and controls. The perforin and granzyme content of NK cells were also reduced; seven of 10 patients had decreased perforin, and four of 10 had decreased granzyme content ($P < 0{\cdot}00001$ and $P = 0{\cdot}0057$). In addition, NK cell-mediated cytotoxicity was reduced in five of 10 patients ($P < 0{\cdot}001$).

B-cell percentage and absolute numbers were decreased in five out of 10 patients (compared to age-matched references). In these five patients the percentage of CD5-positive and CD10-positive B-cells were decreased, as compared to age-matched controls (data not shown).

Antigen proliferation in response to tetanus was lower in four of eight patients evaluated with FA ($P = 0{\cdot}008$) while responses to candida and PHA were within normal limits. Cytotoxic T cell function was also reduced in three of eight patients evaluated ($P < 0{\cdot}0001$). Immunoglobulin G levels were tested in four patients and were normal despite low B cell numbers.

Discussion

Studies of immune function in children and adults with FA mostly consist of reports on small numbers of patients with FA, with limited evaluation of their lymphoid function. In our cohort of 10 patients with FA we report a significant, novel, and previously unappreciated abnormality in cytotoxic T cell function, despite normal quantitative evaluation, and significantly reduced NK cell number and function in the majority of these children. Moreover, our cohort of patients demonstrated a quantitative abnormality in the B cell compartment, with significantly lower absolute number of B cells relative to age-based normals. As B and NK cells mature in the bone marrow, they may probably be most affected as bone marrow failure develops.

Previously Froom *et al* (1987) reported low NK cell activity with normal NK cell numbers in two patients with FA and their family members, suggesting a potential intrinsic defect in NK cell activity associated with FA (Froom *et al*, 1987). Two subsequent studies showed decreased NK cell cytolytic function in patients with FA and their family members but did not provide quantitative assessment of NK cell subsets (Hersey *et al*, 1982; Lebbe *et al*, 1993). Petridou and Barrett (1990) demonstrated decreased NK cell subsets in obligate heterozygotes, without functional assessment. Our data extends the observation of blunted cytotoxic function in NK cells and demonstrated decreased NK cell perforin and granzyme expression with concomitant quantitative deficits of NK cell numbers in individuals with FA. This immunological defect in the NK-cell compartment is especially interesting as it may critically alter immune surveillance with regard to neoplastic cells in this patient population with known predisposition to DNA damage and malignancy.

A previous study in FA patients has shown lower lymphoproliferative responses to PHA and pokeweed mitogens, compared to controls, implying that these patients may have a general immune defect (Suzergoz *et al*, 2008). Further, this study showed that PBMCs of individuals with FA responded poorly to stimulation with tetanus toxoid and a purified protein derivative of mycobacterium, but not cytomegalovirus (CMV) antigen, compared to controls. Abnormal PHA induced proliferation was also demonstrated in another study of heterozygote family members of patients with FA (Petridou & Barrett, 1990). Other reports have demonstrated normal lymphocyte proliferation to mitogens and antigens (Lebbe *et al*, 1993). Our cohort confirmed poor response to tetanus toxoid in patients with FA, however demonstrated normal response to candida and PHA. Together these data suggest that patients with FA may have lower activation and proliferation capabilities and therefore increased susceptibility to some, but not all infections.

Previously Castello *et al* (1998) had demonstrated a quantitative defect in the B cell compartment, but also reported a decrease in CD4+ T lymphocytes, which we did not detect

in our cohort (data not shown). A functional evaluation of the B cell compartment with immunoglobulin G levels in our group was normal although only four of ten patients were evaluated, and testing for other immunoglobulin subtypes was not performed. This finding supports the results from a previous study of patients with FA in whom immunoglobulin levels were reported to be within the normal range, (Roxo *et al*, 2001) but are in contrast with another report of hypogammaglobulinaemia in a patient with FA (Sarwar & Hamadeh, 2009). Grossly normal immunoglobulin levels, however, do not guarantee immunoglobulin diversity, and evaluation of titres to specific antigens e.g. tetanus, diphtheria, varicella, etc. would be of interest.

Half of our patients had reduced numbers of B cells. Interestingly, the phenotype of the remaining B-cells was nearly identical in these patients (all in the paediatric age range), showing reduced percentage of CD5 and CD10 expressing B cells. This particular B-cell phenotype would be expected in adults, especially with advancing age (as mentioned, CD10 is a marker of recent bone marrow emigrants) (Bleesing, 2004). These findings raise an important question, whether there is premature senescence affecting B-cells in patients with FA *versus* decreased production. In either case the response to new antigens (e.g. response to HPV vaccination) could be blunted. Follow-up studies are in progress to assess (and enumerate) B-cell precursors in different stages of development (in bone marrow). We are also evaluating B-cell ‘reserve’ in patients with FA, linked to age and marrow failure.

Fanconi anaemia is not unique among inherited bone marrow failure syndromes with regards to immunological dysfunction. Patients with Dyskeratosis congenita have been observed to have lymphopenia with very low B and NK cell numbers, hypogammaglobulinaemia and decreased T cell proliferation to mitogens. Although numbers reported are small, there is a suggestion that in at least a subgroup of these patients, immune deficiencies may precede manifestations of bone marrow failure (Jyonouchi *et al*, 2011). Patients with Shwachman Diamond syndrome have also been reported to have immunological dysfunction with defects in chemotaxis and neutropenia seen in most patients, as well as reports of subsets of patients with at least one of the following: hypogammaglobulinaemia, decreased B, T and NK cells or decreased T cell proliferative responses (Dror, 2005). Finally, there are reports of immune dysfunction in patients with Diamond-Blackfan anaemia as well, with case reports of decreased T cell proliferative responses as well as decreased specific titres and immunoglobulin levels (Khan *et al*, 2011). Given the heterogeneous nature of these disorders and their clinical manifestations, future collaborative and prospective longitudinal studies are needed to further characterize these immune deficiencies and determine if they are fixed at birth or, rather, progressive and secondary to bone marrow failure.

Our data provide evidence of immune dysfunction in a significant number of patients with Fanconi anaemia, particularly involving NK cells. Further understanding of this immune dysfunction is important in the context of high risk of neoplasia in patients with FA, especially viral-associated malignancies such as HPV-related gynaecological or HNSCCs. Although the extent to which HNSCCs are associated with HPV in individuals with FA remains unclear, the role of immune deficiency with regards to susceptibility or chronic persistent infection may be substantial. In addition, an abnormal NK cell compartment will also have a potential impact on immune surveillance and development of these viral-

associated malignancies. Better understanding of this interplay would allow for a more thoughtful approach to vaccination in this population in order to prevent infection and ultimately reduce their risk of squamous cell cancers.

Response to vaccination should be evaluated in the context of each patient's immune competency. Additional studies in larger number of patients with FA will further characterize these immune deficiencies and define the time course of their development. These will further help correlate the findings with development of progressive marrow failure and malignancies, and may offer avenues for possible interventions in this high-risk patient population.

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Table I

Characteristics of patients with Fanconi anaemia.

Pt	Age (years)	Sex	Complementation	WBC ($\times 10^9$/l)	ANC ($\times 10^9$/l)	ALC (normal range) ($\times 10^9$/l)	Hb (g/l)	Plt ($\times 10^9$/l)
1	3	F	A	5·7	1·43	3·63 (3·00–9·50)	1·2	36
2	3	F	A	6·0	1·2	4·02 (3·00–9·50)	1·1	51
3	4	F	A	4·0	0·92	3·00 (2·00–8·00)	0·8	19
4	5	F	C	4·0	0·44	3·16 (2·00–8·00)	0·9	21
5	6	F	A	4·8	1·44	2·69 (1·50–7·00)	1·3	128
6	7	M	Non-A, B, C, D2, G, E, F, L	6·6	2·05	4·09 (1·50–7·00)	1·2	248
7	7	M	A	2·9	0·12	2·64 (1·20–5·20)	0·7	116
8	15	F	A	2·7	0·89	1·59 (1·50–6·50)	1·1	52
9	16	F	A	4·0	1·56	2·00 (1·20–5·20)	1·3	129
10	16	F	A	3·1	1·58	1·09 (1·50–6·50)	1·2	165

Pt, patient; ANC, absolute neutrophil count; ALC, absolute lymphocyte count; Plt, platelet count; Hb, haemoglobin concentration.

Table II

Quantitative and qualitative immune function in patients with Fanconi anaemia.

	Assay (normal range)										
Patient	**Absolute CD3 ($\times 10^9$/l)**	**Absolute CD19 ($\times 10^9$/l)**	**Absolute CD16/56 ($\times 10^9$/l)**	**% perforin /NK cells**	**% granzyme/NK cells (77–95)**	**NK Cytotoxicity (>3·1 lu)**	**IgG (g/l)**	**Tetanus (>15 289) cpm**	**Candida (>15 289) cpm**	**PHA (>138 000) cpm**	**CTL (>6·0 lu)**
1	1·96 (0·90–4·50)	1·44 (0·20–2·10)	0·36 (0·10–1·00)	83 (81–91)	85	17·9	NA	2458	21 565	232 358	6·0
2	2·613 (0·90–4·50)	1·085 (0·20–2·10)	0·281 (0·10–1·00)	89 (81–91)	93	10·4	NA	795	45 288	188 246	3·5
3	1·812 (0·90–4·50)	0·397 (0·20–2·10)	0·248 (0·10–1·00)	88 (81–91)	86	26	NA	7861	143 552	NA	3·3
4	2·554 (0·90–4·50)	0·147 (0·20–2·10)	0·115 (0·10–1·00)	68 (81–91)	81	3	103 (56–130·7)	NA	NA	NA	NA
5	3·476 (0·90–4·50)	0·62 (0·20–1·60)	0·176 (0·09–0·90)	58 (81–91)	64	1·6	NA	24 972	29 663	122 319	7·4
6	2·48 (0·70–4·20)	1·271 (0·20–1·60)	0·287 (0·09–0·90)	73 (81–91)	86	7·3	NA	60 176	99 896	233 265	9·4
7	1·80 (0·70–4·20)	0·065 (0·20–1·60)	0·077 (0·09–0·90)	65 (81–91)	70	0·6	122 (59·8–137·9)	302	2124	187 390	0·5
8	1·372 (0·80–3·50)	0·023 (0·20–0·60)	0·09 (0·07–1·20)	71 (81–91)	80	2·6	120 (68–153·1)	12 181	128 591	199 078	27·5
9	1·732 (0·80–3·50)	0·019 (0·20–0·60)	0·112 (0·07–1·20)	69 (86–98)	76	0·2	93·4 (72·4–161·1)	18 418	225 410	213 916	73·0
10	0·528 (0·80–3·50)	0·036 (0·20–0·60)	0·042 (0·07–1·20)	48 (86–98)	67	8	NA	NA	NA	139 295	NA
Z score mean (SD)	−0·13 (0·9)	−1·64 (2·27)	−1·06 (0·61)	−2·98 (2·55)	−0·8 (1·04)	N/A	N/A	N/A	N/A	N/A	N/A
P value	0·67	0·048	0·0002	<0·00001	0·0057	<0·0001	N/A	0·0008	0·1833	0·1833	<0·0001

NK, natural killer; IgG, immunoglobulin G; PHA, phytohemagglutinin; CTL, cytotoxic T lymphocyte function; lu, lytic units; cpm, counts per minute; N/A, not applicable; SD, standard deviation; NA, not available.