British Journal of Haematology, 2001, **114**, 712–717

Immune function in patients with Shwachman–Diamond syndrome

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Received 2 March 2001; accepted for publication 9 May 2001

Summary. Shwachman–Diamond syndrome (SDS) is an inherited multisystem disorder characterized by exocrine pancreatic dysfunction and varying degrees of cytopenia. In addition, various immunological abnormalities have been noted. To clarify the issue of immunological competence or incompetence in SDS, we prospectively studied immune function in 11 patients with SDS. Seven suffered from recurrent bacterial infections and six from recurrent viral infections. Varying degrees of impairment were readily identified. All patients had neutropenia; total lymphocyte counts, however, were normal in all except one patient. Nine patients had B-cell defects comprising one or more of the following abnormalities: low IgG or IgG subclasses, low percentage of circulating B lymphocytes, decreased *in vitro* B-lymphocyte proliferation and a lack of specific antibody production. Seven out of nine patients studied had at least one T-cell abnormality comprising a low percentage of total circulating T lymphocytes or $CD3^+/CD4^+$ cell subpopulations or decreased *in vitro* T-lymphocyte proliferation. Five out of six patients studied had decreased percentages of circulating natural killer cells. Moreover, neutrophil chemotaxis was significantly low in all the patients studied. These data point to a major immunodeficiency component in SDS that places patients at heightened risk of infections, even if neutrophil numbers are protective. This finding broadens the definition of the syndrome substantially: it suggests that the SDS marrow defect occurs at the level of an early haematological–lymphocytic stem cell or that a combined marrow and thymic stromal defect accounts for the aberrant function of haematopoietic and lymphopoietic lineages.

Keywords: Shwachman–Diamond syndrome, immunodeficiency, marrow failure, inherited.

Shwachman–Diamond syndrome (SDS) is an inherited multisystem disorder characterized by exocrine pancreatic dysfunction, varying degrees of cytopenia and metaphyseal dysplasia (Mack *et al*, 1996; Smith *et al*, 1996; Ginzberg *et al*, 1999). The molecular basis for the disease is unclear. We have previously shown that bone marrow from patients with SDS is characterized by a decreased frequency of $CD34^+$ cells and marrow $CD34^+$ cells have a reduced ability to form haematopoietic colonies *in vitro* (Dror & Freedman, 1999). Bone marrow failure in SDS appears to be mediated by increased apoptosis as the central pathogenetic mechanism. This increased propensity for apoptosis is linked to increased expression of the Fas antigen and hyperactivation of the Fas signalling pathway (Dror & Freedman, 2001).

Although immunological abnormalities are not traditionally perceived as part of the disease complex, patients with SDS are prone to recurrent infections even in the face of protective neutrophil counts, according to case reports describing immunological defects in patients with the syndrome (Hudson & Aldor, 1970; Aggett *et al*, 1979; Sacchi *et al*, 1982; Kornfeld *et al*, 1995; Maki *et al*, 1978). Neutrophil and monocyte chemotaxis was studied using old methods and usually found to be abnormally depressed (Hudson & Aldor, 1970; Aggett *et al*, 1979; Sacchi *et al*, 1982; Repo *et al*, 1987).

To address the immune competence or incompetence in SDS definitively, we prospectively evaluated the immunological profile of 11 patients with SDS using up-to-date technology. We discovered a major immunodeficiency

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component that places patients at heightened risk of bacterial, fungal and viral infections, even if neutrophil numbers appear protective.

PATIENTS AND METHODS

Patients. The study was conducted from April 1997 to June 1999. Fourteen patients fulfilled the phenotypic criteria for the diagnosis of SDS previously established at our institution (Ginzberg *et al*, 1999) and were asked to participate in the study during their annual visit to the haematology clinic (Table I). The unique patient number (UPN) identification correspond to those used in our previous research reports on these patients (Dror & Freedman, 1999). Eleven patients or their parents gave their consent for the studies and three declined. All the patients have been seen by both the Haematology and Gastroenterology Programs at least once a year, even if the haematological or the pancreatic manifestations were mild. None had received transfusions or haematopoietic growth factors. Two patients (UPN1 and UPN14) had marrow cytogenetic abnormalities of clonal i(7q) and del(20q) respectively (Dror *et al*, 1998). Patient ages ranged from 2 to 18 years (mean, 10·5 ± 5·6). All patients were free of clinically apparent infection at the time of analysis.

Serum immunoglobulins and IgG subclasses. Serum levels of IgG, IgM and IgA were measured by laser nephelometry (Rose *et al*, 1992). Serum IgE concentrations were determined by radioimmunoassay with the IgE paper radioimmunosorbent test (PRIST) kit (Pharmacia Diagnostics, Dorval, Quebec, Canada). The IgG subclass levels from IgG1 to IgG4 were determined using the radial immunodiffusion (RID) kit (The Binding Site, San Diego, CA, USA). Values were compared with age-related normal controls obtained in our laboratory (Dalal *et al*, 1998). Levels below two standard deviations were considered abnormal.

Specific antibody titres. Antibody titres to poliovirus subtypes 1, 2 and 3 were measured using a microneutralization technique (normal value $> 1{:}8$), and to mumps and measles using immunofluorescence (Dalal *et al*, 1998). Antibodies to tetanus toxoid were determined using an enzyme-linked immunosorbent assay (normal value $> 0{\cdot}05$ U/ml). Isohaemagglutinin titres were measured by a standard indirect Coombs' methodology (normal values: anti A $\geqslant 1{:}16$, anti B $\geqslant 1{:}32$).

Lymphocyte subpopulations. Mononuclear cells were separated from 5 to 10 ml volumes of EDTA anticoagulated blood by red cell lysis using the Qprep lysing kit (Coulter, Hialeah, FL, USA) as previously described (Loebstein *et al*, 1997). Lymphocyte subpopulations were determined by two-colour flow cytometric analysis with a fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin antibody and a panel of directly conjugated monoclonal antibodies (Beckman Coulter, Fullerton, CA, USA), as previously described (Bentur *et al*, 1991). Appropriate isotype controls were used each time a patient sample was analysed. Analyses were performed on a Coulter EPICS V flow cytometer (Coulter, Hialeah, FL, USA). Results were expressed as percentages of positive cells within the lymphocyte gate.

Lymphocyte proliferative responses. Mononuclear cells were isolated from freshly heparinized peripheral blood over a Ficoll-Hypaque gradient. Lymphocyte proliferative responses to several mitogens [phytohaemagglutinin (PHA), concavalin A (Con A), pokeweed mitogen (PWM), staphylococcal-antigen Cowan strain I (SAC), and staphylococcal protein A (SPA)] were determined as counts per minute (CPM) by tritiated thymidine incorporation using the microtitre plate technique as previously described (Loebstein *et al*, 1997). All assays were performed in triplicate and results compared with those of simultaneously performed assays of normal control samples. Results were expressed as the stimulation index (SI), as follows:

$$\mathrm{SI} = [\mathrm{CPM\ (stimulated)} - \mathrm{CPM\ (background)}]/\mathrm{CPM\ (background)}$$

Table I. General and haematological characteristics of patients with Shwachman–Diamond syndrome.*

UPN	Age at study (years)	Sex	Metaphyseal dysostosis	Short stature	Recurrent bacterial infections	Recurrent viral infections	Neutrophil count ($\times 10^9$/l)	Lymphocyte count ($\times 10^9$/l)
1	7	M	–	+	+	+	0·4†	4·5
3	14	M	Mild	+	–	–	1·3†	3·0
4	16	F	Mild	–	–	–	0·4†	3·2
5	13	F	Severe	+	+	+	1·5	3·19
6	2	M	–	–	+	+	0·6†	3·1
7	14	F	UD	+	–	–	0·3†	1·8
8	18	M	Severe	+	+	+	1·6	1·7
9	6	F	Moderate	+	–	–	2·5	4·2
10	1	F	UD	+	+	+	1·0†	6·2
11	8	M	Moderate	+	+	+	0·7†	2·7
14	17	M	–	–	+	–	0·4†	0·9†

*All patients had pancreatic dysfunction.
†Low for age.
M, male; F, female; UD, undetermined; UPN, unique patient number.

Neutrophil under-agarose chemotaxis assay. Neutrophils were isolated from anticoagulated whole blood using Neutrophil Isolation Media (Cardinal Associates, Sante Fe, NM, USA), resuspended in Hanks balanced salt solution (HBSS; Gibco/BRL) and used immediately. Assays of neutrophil migration along a chemotactic gradient (fMLP) were performed as described by Repo *et al* (1978) with minor modifications. The difference between neutrophil migration towards fMLP and HBSS was calculated, along with the mean distance that 7×10^5 neutrophils migrated. All experiments were done in triplicate.

Neutrophil microchemotaxis chamber assays. Neutrophils isolated as described above were labelled with 1·5 μmol/l calcein-AM for 20 min at 37°C. Microplate chambers of 96 wells with accompanying 3-μm pore polycarbonate filters from Neuro Probe (Cabin John, MD, USA) were used. Chemoattractant (1 μmol/l fMLP) was placed in the lower chamber and 25 μl of 4×10^6 neutrophils/ml were placed on top of the chamber. Following 1 h incubation at 37°C, the fluorescence of each well was determined using a Cytofluor 2300 fluorescent plate reader. The number of neutrophils that migrated was quantified using a standard curve (cell number versus fluorescence) constructed separately for each set of experiments. All assays were done in triplicate.

Statistical analysis. Results of the tests were compared with reference values previously defined by our institution according to age. Student's *t*-test was used to determine the statistical significance of differences in neutrophil migration distances between patients and controls under the agarose assay, and differences in the numbers of migrating neutrophils in the microchemotaxis chamber assay. Fisher's test was used to determine statistical differences in immunological function of patients with varying severity of haematological or pancreatic function. *P*-values $< 0{\cdot}05$ were considered significant.

RESULTS

Infection complications

Clinical and haematological characteristics of the patients are summarized in Table I. All patients had persistent or intermittent neutropenia. Six patients had a history of recurrent viral infection including recurrent upper respiratory tract infection, gastroenteritis and short episodes of unexplained fever. Seven had recurrent bacterial infection including otitis media and pneumonia. Except for one severely neutropenic patient with a history of pneumonia, no one experienced life-threatening infectious complications during the year before entering the study. Viral infections tended to be frequent in the first 5 years of age, then gradually decreased. Bacterial infections were also more common at an early age. However, severely neutropenic patients (neutrophil $< 0{\cdot}5 \times 10^9$/l) still experienced bacterial infections even after the first 5 years of age.

Humoral immunity

Nine patients had at least one abnormality of B-cell numbers or function (Table II). Three patients had low total serum IgG levels and one had abnormally low IgA levels. Of the eight patients studied, serum IgG1, IgG2, IgG3 were low in three, one and two patients respectively. Serum IgM, IgG4, C3, C4 and total haemolytic complement activity were within reference ranges in all patients. Specific antibodies to tetanus, mumps, measles, polioviruses 1, 2 and 3, rubella and pertussis were normal in six of the seven patients in which it was tested; one patient did not have antibodies to mumps despite vaccination. Anti-A isohaemagglutinin levels were low in two of eight patients with blood type B or O, and anti-B isohaemagglutinin levels were low in six out of eight patients with blood type A or O. Abnormally low proliferation in response to SAC was observed in one out of eight patients studied. Percentages of circulating B-lymphocytes ($CD19^+$ and $CD20^+$ cells) were decreased in one out of six patients studied.

Cellular immunity

Total lymphocyte counts were normal in all patients except one (UPN14), who had persistent mild to moderate lymphopenia ($0{\cdot}89–1{\cdot}4 \times 10^9$/l). Of nine patients studied, seven had at least one documented abnormality in T-cell numbers or function (Table III). The percentage of total T lymphocytes ($CD2^+$ cells) was decreased in one out of six patients. $CD3^+/CD4^+$ cells were decreased in one out of six patients. An inverse CD4 to CD8 ratio was found in three cases (0·5, 1·0 and 1·3 respectively; normal 1·5–3). Of nine patients studied, abnormally low lymphocyte proliferation responses to PHA, Con A, PWM and SPA were found in none, two, four and one patient(s) respectively.

Natural killer cells

Five out of six patients studied had abnormalities related to the percentage of circulating natural killer (NK) cells. Decreased $CD56^+$, $CD16^+$ or $CD16^+/CD56^+$ cells were found in one, three and five patients respectively (Table III).

Neutrophil chemotaxis

Mean of the migration distances under agarose in response to fLMP was reduced in all six patients studied (mean ± SEM, 87 ± 64) compared with six controls (271 ± 54) ($P = 0{\cdot}05$) (Fig 1A). The mean number of neutrophils from three patients that migrated into the microchemotaxis chamber (12 631 ± 4288) was also significantly lower than controls (55 756 ± 15 386) ($P < 0{\cdot}01$) (Fig 1B).

Correlation between the immunological abnormalities and the gastrointestinal and haematological abnormalities

No obvious correlation between the immunological and the gastrointestinal abnormalities could be identified. Among the patients with pancreatic insufficiency (clinical and laboratory evidence of pancreatic dysfunction), there were three out of five patients with B-cell defects and three out of four patients with T-cell defects. In contrast, among the patients with pancreatic sufficiency (laboratory but not clinical evidence of dysfunction), there were five out of six patients with B-cell abnormalities ($P = 0{\cdot}67$) and four out of five patients with T-cell abnormalities ($P = 0{\cdot}72$). Similarly, no obvious correlation between the immunological

Table II. B-cell characteristics of patients with Shwachman–Diamond syndrome.

UPN	IgG* (g/l)	IgM* (g/l)	IgA* (g/l)	IgE (IU/ml)	IgG1 (g/l)	IgG2 (g/l)	IgG3 (g/l)	IgG4 (g/l)	Anti-A IH ($n \geq 16$)	Anti-B IH ($n \geq 32$)	Anti-mumps immunity	Lymph response to SAC	CD19 cells (%)	CD20 cells (%)
1	7·0†	0·3	0·8	46	3·4†	3·3	0·8	0·6	1:32	1:8†	Negative	Normal	24	24
3	7·5	0·4	2	136	5·2	1·2	0·8	0·1	–	1:32	Positive	Normal	12	13
4	9·7	0·4	1	UD	5·7	3·0	0·6	0·2	1:128	1:4†	Positive	Normal	10	9
5	13·2	0·3	1·4	UD	UD	UD	UD	UD	UD	UD	Positive	UD	UD	UD
6	6·1†	0·3	0·2†	22	2·5†	1·4	0·2†	0·7	–	0†	UD	UD	UD	UD
7	16·5	1·3	2·7	UD	12	3·8	1·7	0·6	1:16	1:16†	Positive	Normal	15	15
8	4·8†	0·3	0·7	UD	3·2†	0·8†	0·3†	0·1	1:16	1:16†	Positive	Low	1·2†	1·2†
9	16·8	0·5	1·2	UD	11·7	5·1	0·8	0·9	1:2†	–	Positive	Normal	UD	UD
10	8·7	0·4	0·3	UD	10·4	3·5	0·7	0·7	1:2†	–	Positive	Normal	UD	UD
11	9·8	0·3	0·7	3	6	1·8	0·6	0·6	1:16	1:16†	UD	Normal	9·3	10
14	8·2	0·4	11·1	4·7	5·5	2·4	0·4	0·1	1:32	1:32	UD	Normal	UD	UD

*Our institutional reference values for the immunoglobulin levels are:

Age (years)	IgG (g/l)	IgA (g/l)	IgM (g/l)
0–0·5	2·7–16·6	0–0·1	0–2·2
0·5–1	5·3–16·8	0·2–1·2	0·3–2·2
1–4	5·9–17·2	0·3–2·4	0·3–2·2
4–7	5·9–17·2	0·4–3·7	0·5–3·1
7–10	6·7–17·3	0·4–3·7	0·5–3·1
10–14	8·2–18·6	0·6–4·3	0·5–3·6
> 14	8·4–18·7	0·6–4·2	0·2–3·9

†Low for age.
SAC, Staphylococcal-antigen Cowan strain I; Lymph, lymphocyte; UD, undetermined; UPN, unique patient number; IH, isohaemaglutinin.

Table III. T and natural killer cell characteristics of patients with Shwachman–Diamond syndrome.

UPN	Lymph response to PHA	Lymph response to ConA	Lymph response to PWM	Lymph response to SPA	CD2 cells (%)	CD3 cells (%)	CD3/ CD4 cells (%)	CD3/ CD8 cells (%)	CD4:CD8 ratio	CD56 cells (%)	CD16 (%)	CD16/ CD56 cells (%)
1	Normal	Normal	Low	Low	73*	71	45	21	2·2	2*	3*	1*
3	Normal	Low	Low	Normal	82	72	39	27	1·5	13	13	13
4	Normal	Normal	Normal	Normal	88	85	44	33	1·3*	6·3	7	4*
5	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
6	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD	UD
7	Normal	Normal	Normal	Normal	82	79	37	38†	1*	10·2*	4·3*	3·5*
8	Normal	Normal	Normal	Normal	95†	94†	32*	60†	0·5*	7·3	4·5*	1·8*
9	Normal	Normal	Low	Normal	UD	UD	UD	UD	UD	UD	UD	UD
10	Normal	Normal	Normal	Normal	UD	UD	UD	UD	UD	UD	UD	UD
11	Normal	Normal	Normal	Normal	88	86†	57	25	2·3	2·9	2·3	1·5*
14	Normal	Low	Low	Low	UD	UD	UD	UD	UD	UD	UD	UD

*Low for age.
†High for age.
PHA, phytohaemagglutinin; ConA, concavalin A; PWM, pokeweed mitogen; SPA, Staphylococcal protein A; Lymph, lymphocyte; UD, undetermined; UPN, unique patient number.

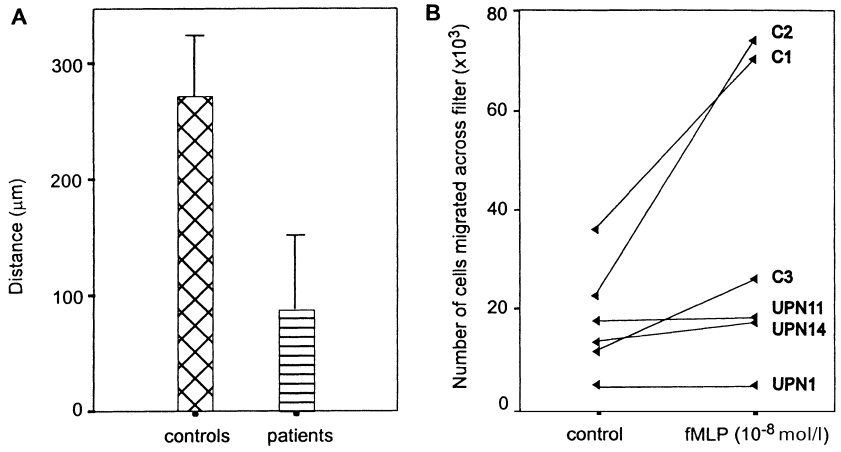

Fig 1. Neutrophil chemotaxis studies. (A) Chemotaxis migration toward fMLP (10^{7} mol/l) as determined by the agarose assay (mean ± SEM, $P = 0{\cdot}05$). Patients studied were UPN 1, 3, 4, 5 and 14. (B) Chemotatic migration toward fMLP (10^{-8} mol/l) as determined using the microchemotaxis chambers (C1–C3, control subjects; P1–P3, patients).

and the haematological abnormalities could be identified. Among patients with pancytopenia (three affected lineages), five out of six patients had B-cell defects and five out of six patients had T-cell defects. In contrast, among patients with one or two lineages affected, three out of five patients had B-cell defects ($P = 0{\cdot}33$) and two out of three patients had T-cell defects ($P = 0{\cdot}58$).

DISCUSSION

We previously showed that the haematopoietic progenitors of SDS are intrinsically flawed, with faulty proliferative properties (Dror & Freedman, 1999) and increased apoptosis mediated through the Fas pathway (Dror & Freedman, 2001). Morbidity and mortality related to infection in SDS patients have usually been attributed to neutropenia. However, concerns about immunological function were raised in several case studies (Hudson & Aldor, 1970; Aggett *et al*, 1979; Sacchi *et al*, 1982; Repo *et al*, 1987; Kornfeld *et al*, 1995; Maki *et al*, 1978).

These reports prompted us to address the immunological profile of our SDS patient population carefully so that definitive conclusions could be drawn about the risk of infection. This study confirms not only that the haematological dysfunction is very broad, but also that lymphopoietic functional capacity is commonly compromised. In addition to consistent neutropenia, we found several prevalent immune deficiencies that might contribute substantially to the infectious complications that occur frequently in these patients. Although the immunodeficiency pattern varied, several defects in T lymphocytes, B lymphocytes, natural killer (NK) cells and neutrophils were demonstrated. Of our SDS patients, 81% (9 out of 11 patients) had abnormalities related to B-lymphocyte numbers or function and 78% (7 out of 9 patients) had abnormalities related to T-lymphocyte numbers or function. Moreover, for the first time we demonstrated abnormalities related to NK cells, as 83% (5 out of 6 patients) of our patients had a low percentage of circulating NK cells. In addition, detailed chemotaxis assays confirmed the presence of a crucial neutrophil chemotaxis defect in all the SDS patients tested.

Detailed analysis of the data suggested that a correlation existed between the occurrence of infection complications and immunological abnormalities. For example, Patients UPN1 and UPN8, who had frequent viral and bacterial infections, were thoroughly investigated and indeed had B, T and NK abnormalities. Patient UPN6, who also had frequent viral and bacterial infections, had low total IgG, IgG1 and IgG3, and isohaemagglutinins. Patient UPN14 had chronic sinusitis and lymphopenia.

The underlying molecular defect in SDS remains unclear and thus the mechanisms of the immunological abnormalities are also unknown. It seems probable that the genetic flaw affects the early pluripotent haematological stem cell so that both the lymphoid and the myeloid stem cells are constitutionally impaired. Alternatively, a combined marrow stromal abnormality (Dror & Freedman, 1999) and a thymic stromal defect are possible. As Fas-mediated apoptosis seems to play a major role in the development of the bone marrow failure in SDS (Dror & Freedman, 2001), the same mechanism might apply to the lymphoid progenitors. We have previously shown that the haematological abnormalities in SDS vary among patients (Dror & Freedman, 1999). Here, we demonstrated that the same principle applies to the immunological abnormalities. The reason for the wide variability in phenotype in a presumably autosomal recessive disease can be explained on the basis of additional genetic determinants or co-operative environmental insults.

Several other disorders are characterized by a combination of various degrees of bone marrow failure and immune defects. These include reticular dysgenesis, hyper IgM syndrome, common variable immunodeficiency (Mentzer *et al*, 1977), warts–hypogammaglobulinaemia–infections–myelokathexis (WHIM) syndrome (Gorlin *et al*, 2000), combined immune deficiency associated with congenital neutropenia (Gasparetto *et al*, 1994), cartilage–hair hypoplasia (Makitie *et al*, 1998), dyskeratosis congenita (Hanada *et al*, 1984), among others (Perreault *et al*, 1985; Wetzler *et al*, 1990; Revy *et al*, 2000). The finding that SDS is another syndrome in which both the immunological and haematological systems are impaired broadens its definition and emphasizes the heightened risk of infections in the syndrome.

ACKNOWLEDGMENTS

We thank Shirley Kwong, Deter Petsche and Jim Lim-Yin for technical assistance. This paper was prepared with the assistance of Editorial Services, The Hospital for Sick Children, Toronto, Canada.

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