International Fanconi Anemia Registry: Relation of Clinical Symptoms to Diepoxybutane Sensitivity

By Arleen D. Auerbach, André Rogatko, and Traute M. Schroeder-Kurth

Fanconi anemia (FA) is characterized clinically by a progressive pancytopenia, diverse congenital abnormalities and increased predisposition to malignancy. A variable phenotype makes accurate diagnosis on the basis of clinical manifestations difficult in some patients. Study of cellular sensitivity to the clastogenic effect of DNA cross-linking agents such as diepoxybutane (DEB) has been used to facilitate the diagnosis. Data from DEB-induced chromosomal breakage studies of 328 peripheral blood specimens from patients considered at risk for FA were analyzed using a stepwise multivariate logistic regression, in order to determine which method of representing the data best discriminated between DEB-sensitive (DEB⁺) and DEB-insensitive (DEB⁻) cases. Similar methods were applied to the data from the International Fanconi Anemia Registry (IFAR) to determine whether DEB⁺ and DEB⁻ cases may be considered as distinct clinical entities, and if so, which variables provide the best discrimination between the two groups. We conclude that hypersensitivity to the clastogenic effect of DEB is a useful discriminator for FA. A simplified scoring method for classifying patients on the basis of eight clinical manifestations that are the best predictors for FA is presented. Our data indicate that the clinical diversity in FA is more widespread than previously recognized.

MATERIALS AND METHODS

Patients. Blood samples for DEB-testing by the RU Cytogenetics Laboratory were obtained from patients seen at the RU Outpatient Clinic, or at a referring medical center. In the latter case, samples were sent by overnight express carrier to the RU laboratory. Patients were ascertained on the basis of the presence of congenital malformations known to be associated with FA, hematologic manifestations such as aplastic anemia or leukemia, both malformations and aplastic anemia, or family screening.

The primary source of case material for the IFAR was voluntary physician reporting. The basis for ascertainment of the patients was the same as described for the DEB testing. Once a potential case was identified, an IFAR questionnaire form was completed by the referring physician and copies of laboratory reports and other patient records were obtained. Chromosome breakage studies including testing for hypersensitivity to the clastogenic effect of the

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DNA crosslinking agent DEB were performed at the RU laboratory, Heidelberg, FRG, or elsewhere.\textsuperscript{4} Patients were classified as FA or non-FA based on sensitivity of cultured peripheral blood lymphocytes to DEB-induced chromosomal breakage. Blood specimens from siblings of FA patients were also tested.

**DEB test.** The method for the DEB test has been previously described in detail.\textsuperscript{13} In summary, the culture unit consisted of 0.4 mL of heparinized blood added to 10 mL of RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 15\% fetal bovine serum (Hazelton Research Products, Denver, PA), 1\% 1-glutamine, 1\% penicillin-streptomycin solution (GIBCO) and 1\% phytohemagglutinin (PHA) (Wellcome Diagnostics, Dartford, England), and incubated for 72 or 96 hours at 37\°C in a 5\% CO\textsubscript{2} atmosphere at high humidity. Cultures were set up in duplicate for DEB studies, and a replicate set of cultures established to serve as untreated controls. DEB (Aldrich Chemical Co, Milwaukee), at a final concentration in the medium of 0.1 \mu g/mL, was added to the treated cultures 24 hours after their initiation, thus exposing cells to the chemical for 48 to 72 hours. This concentration was chosen because it induces multiple chromosomal breaks and gaps in FA cells, while having little clastogenic effect on normal cells. The mitotic index was usually not affected by this concentration of DEB. Dilutions were prepared just before addition of DEB to cell cultures. Cultures were harvested after 72 to 96 hours, and chromosome preparations made following standard methods.

Since DEB is a suspected carcinogen with unknown risk, appropriate precautions were taken. Cultures with DEB were handled using latex gloves, and all work was done in a vertical laminar flow hood or a chemical fume hood (for the first part of the harvest procedure). Since DEB is rapidly inactivated by concentrated hydrochloric acid (HCl), all disposable culture bottles and pipettes were rinsed with HCl before being discarded. HCl was added to spent tissue culture medium before disposal, and was used in case of spillage.

Analysis was performed on 50 to 100 Giemsa-stained metaphases from each DEB-treated preparation (25 metaphases in the case of very high breakage). If DEB-induced chromosomal breakage was increased over the normal range, 50 Giemsa-stained metaphases from the untreated preparation were analyzed. To avoid bias in cell selection, consecutive metaphases that appeared intact with sufficient well defined chromosome morphology were selected for study. Each cell was scored for chromosome number and for the numbers and types of structural abnormalities. Achromatic areas less than a chromatid in width were scored as gaps, while exchange configurations, translocations, and dicentric and ring chromosomes were scored as rearrangements. Gaps were excluded in the calculations of chromosome breakage frequencies, and rearrangements were scored as two breaks.

**Discriminant analysis.** A stepwise multivariate logistic regression\textsuperscript{14} was applied in two instances: (a) to determine which method of representing the data in the chromosomal breakage studies best discriminated between DEB\textsuperscript{+} and DEB\textsuperscript{-} cases, and (b) to determine whether DEB\textsuperscript{+} and DEB\textsuperscript{-} cases may be considered as distinct clinical entities, and if so, which variables provide the best discrimination between the two groups. In the second instance, after applying the logistic regression, a method for classifying the patients based on a simplified score was derived.

**RESULTS**

**DEB studies.** A stepwise multivariate logistic regression was performed on data from DEB studies at the RU Cytogenetics Laboratory. The purpose of this analysis was to determine which method of representing the chromosomal data best discriminated between the DEB\textsuperscript{+} and DEB\textsuperscript{-} groups. The independent variable was group (DEB\textsuperscript{+} or DEB\textsuperscript{-}) and the predictors were breaks per cell, percent of cells with breaks, or breaks per aberrant cell, and sex. The results indicated that breaks per cell alone gave absolute discrimination (Table 1). There was no overlap in the range for the DEB\textsuperscript{+} (FA) group compared with the DEB\textsuperscript{-} (non-FA) group (Fig 1). There was no significant difference in DEB-induced chromosomal breakage when male and female FA patients were compared. While most FA patients exhibited multiple chromatid breaks and exchanges in most or all cells analyzed after DEB exposure, approximately 10\% of these patients appeared to have two populations of lymphocytes. In these individuals, the majority of DEB-treated cells examined (from 60\% to 90\%) appeared to have no chromosomal breakage, while the remainder of cells examined in each case exhibited the high number of breaks and exchanges typical of FA patients. The mean DEB-induced chromosomal breakage in these patients ranged from 1.06 to 2.56 breaks per cell, while the mean breakage frequency calculated as breaks per aberrant cell was 6.13, similar to that found in typical FA patients. The DEB-induced chromosomal breakage frequency in the non-FA group was similar to that reported previously for control individuals.\textsuperscript{4}

Analysis of baseline chromosomal breakage in the FA group showed that the frequency in some patients did not differ from that found in normal controls or in the non-FA group. The range of breakage in untreated cells was 0.02 to 1.9 breaks per cell (mean, 0.27) for the FA group and 0 to 0.12 breaks per cell (mean, 0.02) for the non-FA group. The difference in the baseline breakage frequencies for these two groups was not statistically significant, indicating that this is not a useful method for discrimination of FA patients.

**IFAR.** Of the 310 patients referred to the IFAR because they had some clinical findings consistent with a diagnosis of

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**Table 1. Chromosomal Breakage in DEB-Treated Peripheral Blood Lymphocytes**

<table>
<thead>
<tr>
<th>Parametrizations</th>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>Median</th>
<th>SE Mean</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breaks/cell</td>
<td>FA</td>
<td>104</td>
<td>8.96</td>
<td>8.73</td>
<td>0.448</td>
<td>1.30</td>
<td>23.90</td>
</tr>
<tr>
<td></td>
<td>Non-FA</td>
<td>224</td>
<td>0.06</td>
<td>0.04</td>
<td>0.004</td>
<td>0.00</td>
<td>0.36</td>
</tr>
<tr>
<td>Cells with breaks (%)</td>
<td>FA</td>
<td>104</td>
<td>85.15</td>
<td>92.00</td>
<td>1.99</td>
<td>12.60</td>
<td>100.00</td>
</tr>
<tr>
<td></td>
<td>Non-FA</td>
<td>224</td>
<td>5.12</td>
<td>4.00</td>
<td>0.28</td>
<td>0.00</td>
<td>22.00</td>
</tr>
<tr>
<td>Breaks/aberrant cell</td>
<td>FA</td>
<td>104</td>
<td>10.22</td>
<td>9.65</td>
<td>0.043</td>
<td>3.60</td>
<td>24.90</td>
</tr>
<tr>
<td></td>
<td>Non-FA</td>
<td>224</td>
<td>0.99</td>
<td>1.00</td>
<td>0.04</td>
<td>0.00</td>
<td>6.00</td>
</tr>
</tbody>
</table>

Difference between FA and non-FA groups for all the parametrizations: \( P < 0.0001 \) (Mann-Whitney test).

Abbreviations: Min, minimum value; Max, maximum value.
FA, 202 were diagnosed as affected and 108 as unaffected, on the basis of DEB testing. The constitution of this sample, classified by sex, is seen in Table 2. There was no significant difference in the sex ratio in either the DEB* (FA) classified by sex, is seen in Table 2. There was no significant

The clinical manifestations of the FA patients were highly variable, and non-FA patients all had some clinical findings associated with FA. Table 3 shows the number of patients manifesting congenital malformations and/or hematologic abnormalities at the time of referral for DEB testing, in both the FA and non-FA patient groups. Both hematologic abnormalities and congenital malformations were observed at the time of referral in only 39% of FA patients. Thirty percent of FA patients had aplastic anemia only; many of these were originally diagnosed as having acquired aplastic anemia. Twenty-four percent were in the pre-anemic stage and were referred because they had malformations associated with FA, while 7% had neither aplastic anemia or malformations. This latter group was comprised of DEB* siblings of FA probands; most of these individuals have developed hematologic manifestations of FA subsequent to their chromosome breakage studies. Among the non-FA patients, approximately 10% were ascertained because they manifested both aplastic anemia and some malformations associated with FA.

In order to test the clinical basis of the classification of patients by the DEB sensitivity test, a stepwise multivariate logistic regression was performed. The predictors were the 12 congenital abnormalities and five hematologic manifestations that were scored for presence or absence in each of the patients in the IFAR. The frequency of these in the DEB* and DEB- groups is seen in Table 4. The results of the discriminant analysis showed that eight variables best discriminated between the DEB* and DEB- groups (Table 5). Thus it is possible to determine the probability of a patient being DEB* or DEB-, taking into account only the presence or absence of these eight selected clinical markers. This supports the concept that the DEB* and DEB- groups of patients belong to distinct clinical entities.

Since the coefficients of the regression did not differ, in absolute value, from unity, we could derive a simplified scoring method for classifying patients. By adding one for the six abnormalities that had positive coefficients and subtracting one for the two abnormalities that had negative coefficients, the probability of a patient belonging to either of the groups can be predicted. The distribution of this score, which varies from -2 to +6, is given in Table 6. The results show that although the majority of patients referred for testing with a score of 0 or +1 were DEB-, approximately 30% of these individuals were in fact DEB*, and may not have been diagnosed as affected with FA if they had not been tested.
developmental defects. The principal diseases in this group are
the presence of increased chromosomal fragility or cellular
instability in untreated cells. On the other hand, the diag-
nosis of FA has been used by other laboratories in a clastogenic
test for FA. DNA crosslinking agents produce monoadducts
as well as crosslinks when they bind to DNA, and the ratio
of these products differs with different chemical agents. DNA
crosslinking agents also differ in their requirement for meta-
bolic activation, and in their stability both in solution and on
the shelf. These variables can affect the reliability of a par-
ticular chemical when used as a diagnostic test for FA. This
is especially a problem if normal individuals exhibit a
wide range of cellular sensitivity to the agent, as they do to
MMC. We are aware of false positive and false negative
diagnoses with MMC; in our experience the use of DEB for
FA diagnosis appears to be more reliable.

It does not appear to be feasible to rely on sensitivity to
DNA crosslinking agents such as DEB or MMC for detec-
tion of carriers of the FA gene. Although chromosome
breakage frequencies intermediate to those found in FA and
normal controls have been reported for individual obligate
heterozygotes in some families, and the mean chromosomal
breakage in DEB-treated cells from obligate carriers as a
group is higher than that found in the control group, there is
much overlap between carrier and control values for DEB
sensitivity to be used as a carrier test.

The extreme phenotypic diversity associated with FA
makes the availability of a diagnostic laboratory test espe-
cially valuable. The demonstration of clinical differences
between the DEB+ and DEB− patient groups supports the
concept that DEB sensitivity provides a reliable marker for
the diagnosis of FA. Although there was considerable hetero-
geneity in the selection of patients for referral for DEB
testing in this study, with some centers sending samples on all
patients with aplastic anemia under a given age regardless of
the diagnosis of FA. Although there was considerable hetero-
geneity in the selection of patients for referral for DEB
testing in this study, with some centers sending samples on all
patients with aplastic anemia under a given age regardless of
other phenotypic characteristics whereas other centers were
more discriminate in their choice of patients for study, we
feel that the large size of our sample provides an adequate
basis for our analysis. A similar discriminant analysis per-
formed previously, when the IFAR sample consisted of 222
patients, resulted in the same eight variables (see Table 5)
being the best predictors for FA. Thus there is a consistency
in the results obtained when these methods are applied.
Results of a segregation analysis performed on the IFAR
data, with patients classified entirely on the basis of the DEB
marker, also resulted in groups that can be considered as
distinct entities. A monogenic autosomal recessive mode of
inheritance was confirmed for the DEB+ group, while the
DEB− group exhibited an excess of sporadic cases, which
may be due to such factors as new mutation, phenocopies,
and polygenic inheritance. Thus the DEB− group is likely to
be heterogeneous, consisting of genetic and nongenetic enti-
ties.

While there is some evidence for genetic heterogeneity in

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### Table 5. Summary of the Stepwise Logistic Regression

<table>
<thead>
<tr>
<th>Step</th>
<th>Variable</th>
<th>Coeff</th>
<th>SE</th>
<th>IMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Growth retardation</td>
<td>.70</td>
<td>.18</td>
<td>38.47</td>
</tr>
<tr>
<td>2</td>
<td>Birthmarks</td>
<td>.78</td>
<td>.17</td>
<td>27.09</td>
</tr>
<tr>
<td>3</td>
<td>Kidney and urinary</td>
<td>.71</td>
<td>.22</td>
<td>17.56</td>
</tr>
<tr>
<td>4</td>
<td>Microphthalmia</td>
<td>.81</td>
<td>.23</td>
<td>12.80</td>
</tr>
<tr>
<td>5</td>
<td>Learning disabilities</td>
<td>-.06</td>
<td>.23</td>
<td>11.62</td>
</tr>
<tr>
<td>6</td>
<td>Low platelets</td>
<td>.73</td>
<td>.20</td>
<td>7.43</td>
</tr>
<tr>
<td>7</td>
<td>Thumb and radius</td>
<td>.85</td>
<td>.21</td>
<td>15.24</td>
</tr>
<tr>
<td>8</td>
<td>Other skeletal</td>
<td>-.57</td>
<td>.20</td>
<td>8.47</td>
</tr>
</tbody>
</table>

Variable refers to the selected variables that best discriminate FA and non-FA groups.
Abbreviations: Coeff, coefficients; SE, standard errors; IMP, chi-square of improvement.

All but one of the patients with a score equal to or greater than +4 were DEB+ in the chromosome breakage analysis.

### DISCUSSION

FA is one of a number of disorders that have in common the presence of increased chromosomal fragility or cellular
hypersensitivity to mutagenic chemicals, associated with
developmental defects. The principal diseases in this group
include ataxia-telangiectasia (A-T), Bloom syndrome (BS),
xeroderma pigmentosum (XP), and Cockayne syndrome
(CS). Laboratory methods for prenatal and postnatal diag-
nosis are currently available for each of these syndromes. Cells from FA patients are uniquely hypersensitive to the
clastogenic effect of DNA crosslinking agents such as DEB,
and can thus be distinguished from cells from patients with
these other syndromes on this basis.

It is clear from the results of chromosome breakage studies
on patients referred to the IFAR that if baseline breakage
frequencies alone are considered, some FA patients would
not be diagnosed, because of lack of expression of chromo-
some instability in untreated cells. On the other hand,
analysis of DEB-treated cells showed no overlap in the
breakage frequency range for the FA group compared with the
non-FA group, and metaphases exhibiting multiple chromatid
breaks and exchanges were found only in affected

cultures. FA patients with two populations of lymphocytes
were detected when sufficient metaphases were analyzed. It
is possible that a patient could have such a low frequency of
DEB-sensitive cells that they remain undetected, but from
our experience we think this would be a very rare occurrence.
It has been suggested that the diagnosis of FA can be

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### Table 6. Probability of Having Fanconi Anemia Based on the Simplified Score

<table>
<thead>
<tr>
<th>Simplified Score</th>
<th>FA (N)</th>
<th>Non-FA (N)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1</td>
<td>0</td>
<td>5</td>
<td>.00</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>20</td>
<td>.20</td>
</tr>
<tr>
<td>1</td>
<td>27</td>
<td>59</td>
<td>.31</td>
</tr>
<tr>
<td>2</td>
<td>53</td>
<td>18</td>
<td>.75</td>
</tr>
<tr>
<td>3</td>
<td>59</td>
<td>5</td>
<td>.92</td>
</tr>
<tr>
<td>4+</td>
<td>58</td>
<td>1</td>
<td>.98</td>
</tr>
<tr>
<td>Total</td>
<td>202</td>
<td>108</td>
<td></td>
</tr>
</tbody>
</table>
FA, the question of whether the extensive phenotypic variation seen in this syndrome is related to genetic differences has not been adequately addressed. We and others have found affected individuals lacking congenital malformations, originally described as the Estren-Dameshek syndrome, in the same sibships as classical FA patients. Thus, clinical heterogeneity cannot be accounted for solely by genetic heterogeneity.

Diagnosis of FA is further complicated by the occurrence of other disorders, both genetic and nongenetic, which are characterized by many of the clinical features seen in FA. Familial associations of various combinations of radial, renal, cardiac, hearing, growth, skin pigmentation, and hematologic abnormalities have been well documented, and a number of different syndromes delineated. Among these are dyskeratosis congenita, TAR syndrome, Holt-Oram syndrome, Aase syndrome, WT syndrome, Shwachman’s syndrome, and IVIC syndrome. Thumb abnormalities have been reported in a number of patients with Diamond-Blackfan anemia. Patients with the sporadically occurring VATER association have several FA-like congenital malformations, demonstrating the nonrandom association of some of these defects in an etiologically diverse group of patients. In addition, similar malformations are sometimes associated with chromosomal syndromes such as trisomy 18,38 13q deletion, and 4p deletion. Patients with malformations referred for DEB-testing and constituting the DEB group in the IFAR, are probably a heterogeneous group composed of genetic and nongenetic entities. We feel that screening for DEB sensitivity in patients to be treated with bone marrow transplantation for aplastic anemia is a useful method for discrimination of FA cases from other patients manifesting some of the clinical features of the syndrome. Accurate diagnosis of these patients is important for therapeutic decision making. Application of the simplified score method for discrimination of FA cases from other patients referred for DEB testing on the basis of aplastic anemia only were hypersensitive in the test. It is especially important to distinguish these cases, as an accurate diagnosis will influence the choice of therapy. Patients with some forms of aplastic anemia respond to treatment with immunotherapeutic drugs such as antithymocyte globulin (ATG) or cyclosporine A, but there is no evidence of benefit to FA patients from these drugs. On the other hand, patients with FA have a high rate of response to treatment with androgen therapy. It is also extremely important to obtain information regarding DEB sensitivity in patients to be treated with bone marrow transplantation for aplastic anemia. Since FA patients are hypersensitive to all DNA crosslinking agents, they require a modified pretransplant conditioning regimen, with a lower than usual dose of cyclophosphamide.

In conclusion, results of this study indicate that testing for hypersensitivity to the clastogenic effect of DEB is a useful method for discrimination of FA cases from other patients manifesting some of the clinical features of the syndrome. Accurate diagnosis of these patients is important for therapeutic decision making. Application of the simplified score derived from the results of the discriminant analysis performed on the IFAR data base should be useful for the selection of cases to be referred for DEB testing.

ACKNOWLEDGMENT

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International Fanconi Anemia Registry: relation of clinical symptoms to diepoxlybutane sensitivity

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