Effects of Deoxycoformycin in Mice

III. A Murine Model Reproducing Multi-System Pathology of Human Adenosine Deaminase Deficiency

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Adult AKR/J mice were treated with 10 μg/g or 100 μg/g 2′-deoxycoformycin, an adenosine deaminase inhibitor with chemotherapeutic potential. The thymus and adrenal glands were decreased in weight more than any other organ. Histologic and cytofluorographic analyses indicated preferential depletion of peanut-agglutinin–positive, cortical thymocytes, as well as acute, dose-dependent damage to the adrenal cortex and medulla. The effect of 2′-deoxycoformycin on the thymus was proven to be independent of the adrenal glands by use of adrenalectomized mice. Dose-dependent liver necrosis, hemolysis, and leukemoid reactivity were observed. These findings illustrate a differential sensitivity of thymocyte subpopulations and suggest, in addition, preferential sensitivity of certain nonlymphoid tissues to 2′-deoxycoformycin administered *in vivo*. (Am J Pathol 1985, 119:65–72)

ADMINISTRATION of 2′-deoxycoformycin (DCF), an inhibitor of adenosine deaminase, in mice induces metabolic and immunologic alterations similar to those observed in human adenosine deaminase–deficient severe combined immunodeficiency disease (ADA⁻-SCID).[1-4] Pathologic studies of DCF-treated mice have so far been mainly restricted to the lymphoid system[5,6] and to the peripheral blood.[4,6-8]

We have recently reviewed the pathologic findings in 8 autopsy cases of human ADA⁻-SCID and were struck by the previously unreported multisystemic changes observed in this enzymatic deficiency disease involving not only lymphoid and chondroosseous tissues, but also neural, endocrine, and renal systems.[9] Seven of 8 cases showed renal mesangial sclerosis. Six of 8 cases showed adrenal gland sclerosis, and one of three pituitary glands examined showed anterior lobe sclerosis. In addition to the known histopathologic alterations of chondroosseous tissue in ADA⁻-SCID, such as short growth plates and few proliferating and some hypertrophic chondrocytes, two cases displayed necrotic chondrocytes and large amounts of cellular debris. One cerebellum examined showed Purkinje-cell dropout. However, it is difficult to determine whether or not these lesions are directly due to the enzyme deficiency or are indirect consequences mediated by the infections which individuals with this syndrome are prone to incur.

Because DCF administered to mice has already been shown to reproduce the biochemical abnormalities found in human ADA⁻-SCID,[2] we chose to undertake a detailed investigation of the pathology of lymphoid, as well as nonlymphoid, organs in mice treated with this ADA inhibitor. This was also considered of importance because pathologic lesions found in DCF-treated mice might have clinical implications for the use of this agent in human chemotherapy (reviewed in Hirschhorn and Ratech[10]).

Materials and Methods

Adult female AKR/J mice (8 weeks) were purchased from the Jackson Laboratory, Bar Harbor, Maine. DCF was obtained from the Natural Products Branch, De-

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velopmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland. Fluorescein-labeled peanut agglutinin (PNA-FITC) was purchased from Vector Laboratories, Inc., Burlingame, California.

Mice were injected subcutaneously with 10 μg/g DCF, 100 μg/g DCF, or normal saline and killed 2 or 4 days after injection. The organs were weighed; and histologic sections for light microscopy, stained with hematoxylin and eosin, periodic acid–Schiff, or methyl green pyronine were prepared. Heparinized blood was collected from the retroorbital venous plexus prior to sacrifice of the mice on Day 2. It was evaluated for red blood cell (RBC) counts and morphology, as well as white blood cell (WBC) and differential counts.

Adrenalectomy was performed on AKR/J mice anesthetized with ether and nembutal via a posterior surgical approach. Sham-operation mice underwent the same procedure, except that the adrenal glands were left *in situ*.

Thymocytes from normal, adrenalectomized or sham-operation AKR/J mice pretreated with a single subcutaneous injection of 10 or 40 μg/g DCF or normal saline were incubated with peanut agglutinin coupled to fluorescein isothiocyanate (PNA-FITC), dilution 1:20, for 30 minutes on ice, washed three times with Hanks' balanced salt solution containing 5% bovine calf serum, and analyzed by visual counting in a Leitz fluorescent microscope or quantitated by means of a Cytofluorograf (Ortho, Westwood, MA).

Staining of spleen sections for IgM was performed by incubation of frozen sections with rabbit anti-IgM (Miles Laboratories, Elkhart, Ind), washing in Tris buffer (pH 7.6, 0.05 M), and incubation with protein A conjugated to horseradish peroxidase (Zymed, Burlingame, Calif). Staining for peroxidase activity was by addition of 0.1 ml of a solution containing 0.075 g of 3,3'-diaminobenzidine tetrachloride plus 0.1 ml 3% H_2O_2 per 100 ml of Tris buffer.

Results

Hematologic Parameters in Mice Treated With DCF

Total RBC, WBC, and differential counts were determined from heparinized blood taken 2 days after injection of mice with normal saline, 10 μg/g DCF, or 100 μg/g DCF. RBC and WBC values and differential counts in normal, untreated adult AKR/J mice agreed well with those previously reported.[11] Two days after a single injection of 10 μg/g the RBC count fell from 7.8 × 10^6 cells/cu mm to 3.7 × 10^6 cells/cu mm, at which time Burr cells appeared (Figure 1). Treatment with 100 μg/g DCF caused the RBC count to fall further to 1.0 × 10^6 cells/cu mm (Table 1). The WBC count was reduced in mice treated with 10 μg/g DCF from 14.4 to 6.3 × 10^3 cells/cu mm and, paradoxically, increased to 41.8 × 10^3 cells/cu mm in mice treated with 100 μg/g DCF (Table 1). Evaluation of the differential WBC count from Giemsa-stained peripheral blood smears showed that 10 μg/g DCF caused significant lymphocytopenia (26% of control) and a 27% decrease in granulocytes. While 100 μg/g DCF also caused an equivalent lymphocytopenia, there was an eightfold increase in the absolute number of circulating myeloid cells (Table 1).

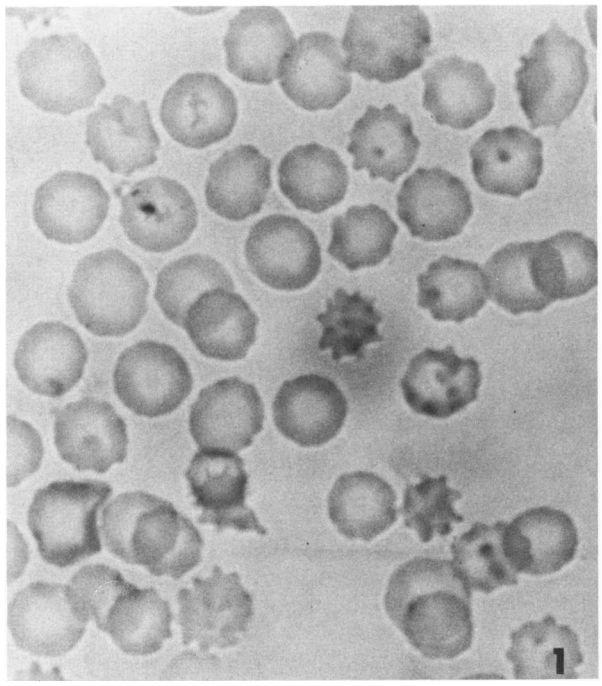

Figure 1—Burr cells appearing in the peripheral blood 2 days after a single injection of 10 μg/g DCF. (× 1000)

Mice injected with DCF excreted a red-brown-tinged urine within 12 hours. This has been shown by others to be due to hemoglobin.[8]

Body and Organ Weights in Mice Treated With DCF

Two days after injection of 10 μg/g DCF there was an approximate 25% reduction in body weight, compared with that of normal saline treated controls, as well as reduction in organ weights for spleen, axillary, and brachial lymph nodes, liver, kidneys, heart, and lungs (12–33%). However, thymus and adrenal glands were more affected than other organs and were reduced by 53% and 55%, respectively. Four days after injection of 10 μg/g DCF, the total body and organ weights remained reduced to about the same levels as at 2 days,

Table 1—Peripheral Blood Analysis in DCF-Treated Mice*

Drug	Control	DCF (10 μg/g)	DCF (100 μg/g)
RBC (no. × 10^6/cu mm)	8.0;7.6	3.2;4.2	1.1;1.0
RBC morphology	Normal	50% Burr cells	90% Burr cells; fragments
WBC (no. × 10^3/cu mm)	13.2;15.6	3.4;9.1	33.0;50.6
Differential count			
Myelocytes (%)	33;28	53;57	91;95
Lymphocytes (%)	67;72	47;44	9;5
Absolute count			
Myelocytes (no. × 10^3/cu mm)	4.4;4.4	1.8;5.2	30.0;48.1
Lymphocytes (no. × 10^3/cu mm)	8.8;11.2	1.6;3.9	3.0;2.5

* Two mice in each group were given 10 μg/g DCF, 100 μg/g DCF, or normal saline and sacrificed after 48 hours. The values listed are from individual mice.

except for the spleen weight, which paradoxically increased almost twofold, compared to that of normal saline-treated controls (Table 2). This has been previously observed in neonatal mice treated with DCF.[2]

Two days after injection of 100 μg/g DCF there was a 32% reduction in body weight, compared with that of normal saline-treated controls. Thymus and adrenal glands were even further decreased than after 10 μg/g DCF (79% and 85%, respectively). With this higher dose (100 μg/g DCF), spleen, lymph nodes, and liver were also disproportionately decreased in weight, compared with other organs (65%, 54%, and 36% reductions). The heart, kidneys, and lungs were least affected (Table 2).

Two additional mice were injected with 100 μg/g DCF, but after 3 days they were noted to be shivering and were cold to touch. They expired by the end of the third day. Their total body and organ weights were similar to those measured in mice given 100 μg/g DCF and sacrificed at 2 days (data not shown).

Histologic Findings in Mice Treated With DCF

Histologic examination of the *spleen* 2 days after injection of 10 μg/g DCF showed little or no lymphoid depletion, compared with normal controls (Figure 2A and B). However, 2 days after injection of 100 μg/g DCF there was a marked cellular depletion of the spleen with only elements of extramedullary hematopoiesis (EMH) remaining (Figure 2C). Four days after injection of 10 μg/g DCF, at the time of maximal splenic enlargement, the lymphoid follicles and periarteriolar sheaths appeared normal, but the cords of Billroth were greatly widened because of increased EMH, as shown by the presence of megakaryocytes and normoblasts. We investigated the distribution of B-cell immunoblasts by staining sections of spleen from DCF treated and control mice for cytoplasmic IgM by the immunoperoxidase technique. The total number of IgM-containing cells was decreased, but their distribution appeared normal.

Injection of 10 μg/g or 100 μg/g DCF was followed by a uniform and progressive lymphocyte depletion in *lymph nodes*. The effect on the paracortex was no greater than on the germinal centers.

The *adrenal glands* showed cortical damage after treatment with 10 μg/g DCF (Figure 3A). Treatment with 100 μg/g DCF also damaged the adrenal medulla (Figure 3B). No inflammatory cell response was evident after either dose.

The *liver* showed spotty necrosis and hemorrhage in pericentral, midzonal, and periportal areas after injection of 10 μg/g DCF. There also were subcapsular infarctions after injection of 100 μg/g DCF. Liver changes were without any inflammatory response.

Table 2—Organ and Body Weights (mg)* in Mice Treated with DCF

Organ weighed	Controls	10 μg/g DCF 2 days (% decrease)		10 μg/g DCF 4 days (% decrease)		100 μg/g DCF 2 days (% decrease)	
Thymus	133	63	(53)	58	(56)	28	(79)
Spleen	65	54	(17)	119	(83)	23	(65)
Axillary and brachial Lymph nodes	24	16	(33)	21	(12)	11	(54)
Adrenal glands	20	9	(55)	9	(55)	3	(85)
Liver	1,501	1,206	(20)	1,060	(29)	966	(36)
Kidneys	320	266	(17)	237	(26)	270	(16)
Lungs	190	136	(28)	161	(15)	138	(27)
Heart	153	135	(12)	131	(14)	147	(4)
Body weight	25,000	19,000	(24)	19,000	(24)	1,700	(32)

* The mice were given normal saline or DCF and sacrificed after 48 hours or 96 hours. The values represent the mean of 4 mice per group given normal saline and of 2 mice for each of the groups given 10 μg/g or 100 μg/g DCF. There was close agreement between the individual values within each group. The percent (%) decrease was calculated with respect to control values. The organ and body weights are in milligrams.

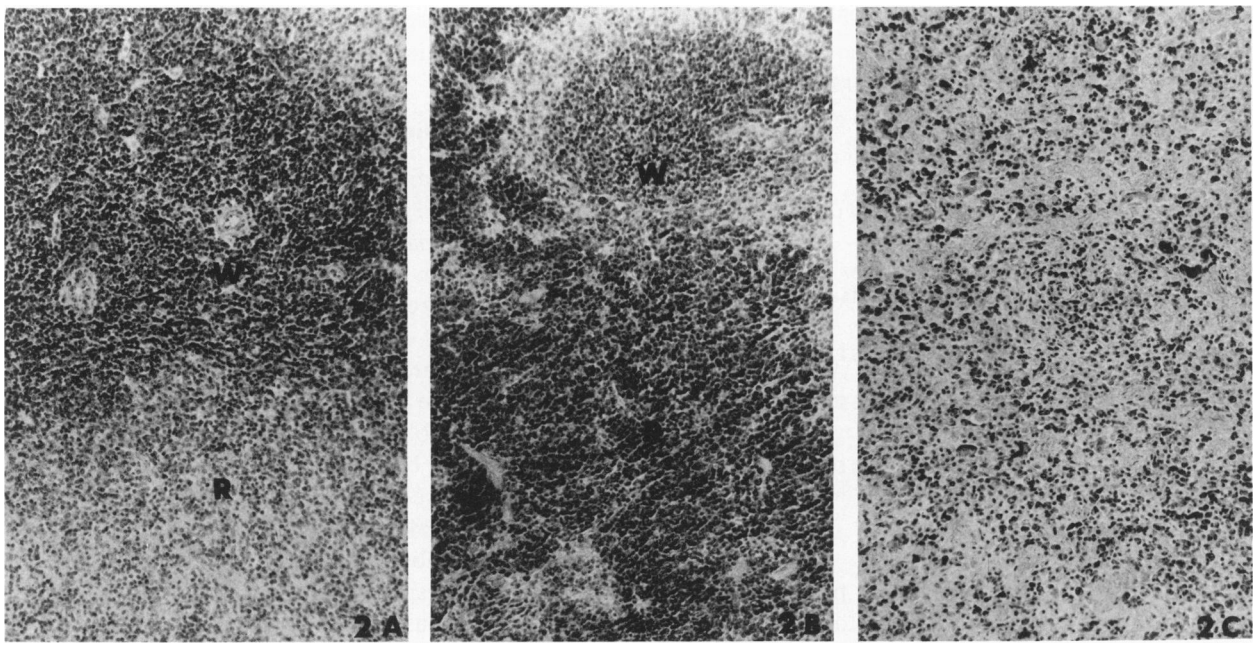

Figure 2—Comparison of normal spleen (**A**) with spleen taken 2 days after injection of 10 μg/g DCF (**B**) or 100 μg/g DCF (**C**). Note the loss of cellularity in white pulp (*W*) and residual hematopoiesis in red pulp (*R*) after 100 μg/g DCF. (×100).

The *kidney* showed acute tubular necrosis after injection of 100 μg/g DCF. Examination of mice given either one or five doses of 10 μg/g DCF during a 2-week period did not show any glomerular or tubular changes by light microscopy.

Examination of the brain showed Bergmann astrocyte proliferation and focal Purkinje cell dropout in the cerebellums of 5 of 6 mice treated with 10 μg/g or 100 μg/g DCF 2 or 4 days previously (Figure 4). Only one of 9 normal control mice had a similar astrocyte proliferation. These changes were not evident in mice examined 10 days after injection of 10 μg/g DCF.

The femurs, lungs, and heart of DCF-treated mice were histologically unremarkable at all dose levels tested.

Effect of DCF on Thymocyte Subpopulations

The lectin PNA binds preferentially to cortical or less mature thymocytes.[12] On thymus sections stained with methyl green pyronine, there appeared to be a greater depletion of cortical than of medullary thymocytes 2 days after DCF treatment. This was further assessed and confirmed by staining sections of thymus with PNA coupled to peroxidase (Figure 5A and B).

PNA-FITC was used in following the course of the disappearance of PNA^+ thymocytes after a single injection of 10 μg/g DCF (Table 3). The nadir of percent PNA-FITC-positive cells, both upon examination in the Cytofluorograf and by direct examination under the fluorescent microscope, was on Day +2 after injection. Gradual recovery was seen to occur after that, although control values had not yet been reached by Day +4.

The distribution of PNA-FITC-stained cells in the Cytofluorograf 5 days after injection of 40 μg/g DCF is illustrated in Figure 6, indicating that recovery to normal is delayed after a high dose of DCF. These are typical results from a study using 5 control mice and 5 experimental mice.

Size, monitored by right-angle scatter versus fluorescence, was examined in PNA-FITC-stained thymocytes from 5 normal control mice and thymocytes from 5 mice given 10 μg/g DCF 2 days previously (Figure 7A and B). Thymocytes from DCF-treated mice were consistently missing a population of large, brightly fluorescent cells and had an excess of small, dimly fluorescent cells, compared with thymocytes from normal controls.

In view of the destructive effect of DCF on the adrenal, it seemed possible that the effect on the thymus was mediated via an acute release of adrenocortical steroids. The effect of DCF on the thymus in adrenalectomized mice was, therefore, also evaluated. The percentages of PNA^+ cells 3 days after DCF injection in adrenalectomized mice were decreased to approximately the same extent as was observed in normal AKR mice (Table 3).

Discussion

The organs with the largest decrease in weight after DCF treatment were the thymus and, unexpectedly, the adrenal glands. The thymus showed a preferential sen-

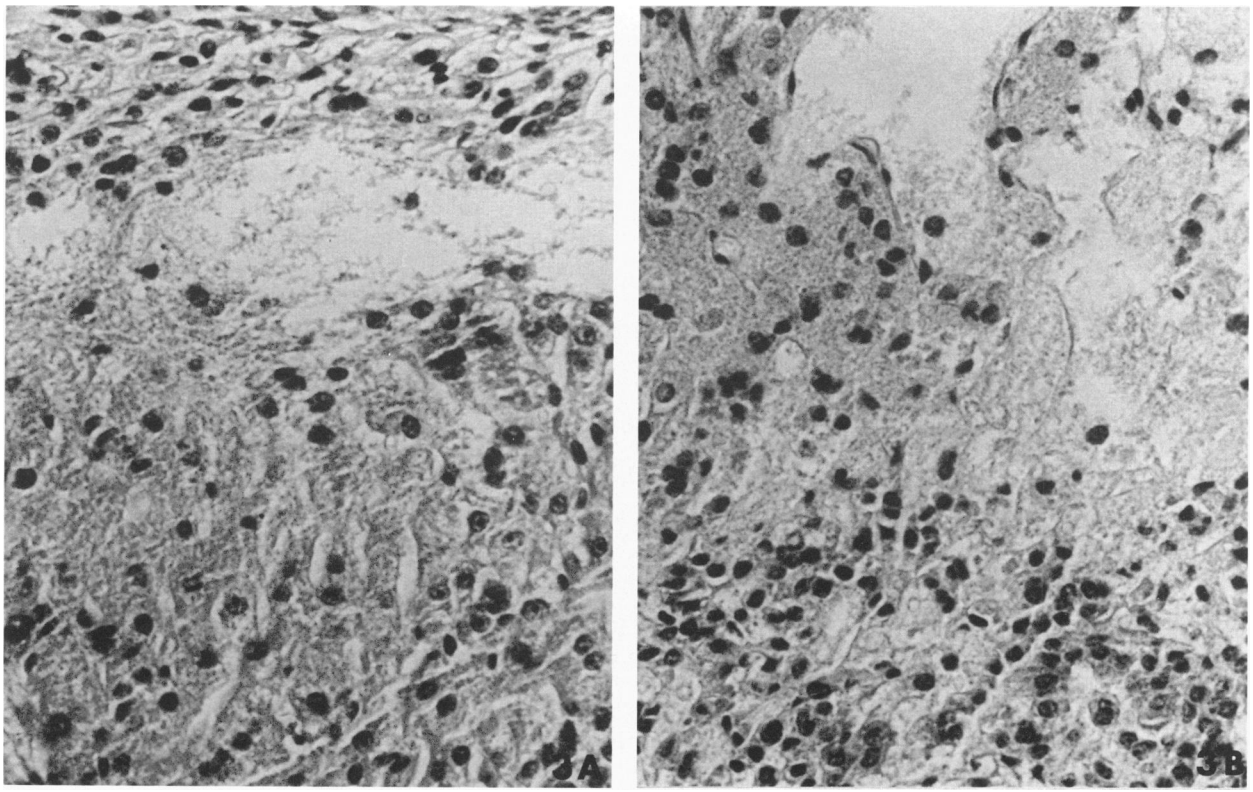

Figure 3—**A**—Focal adrenal gland cortical damage without an inflammatory cell response 2 days after a single injection of 10 μg/g DCF. **B**—Necrosis of the adrenal gland medulla 2 days after a single injection of 100 μg/g DCF. (×300)

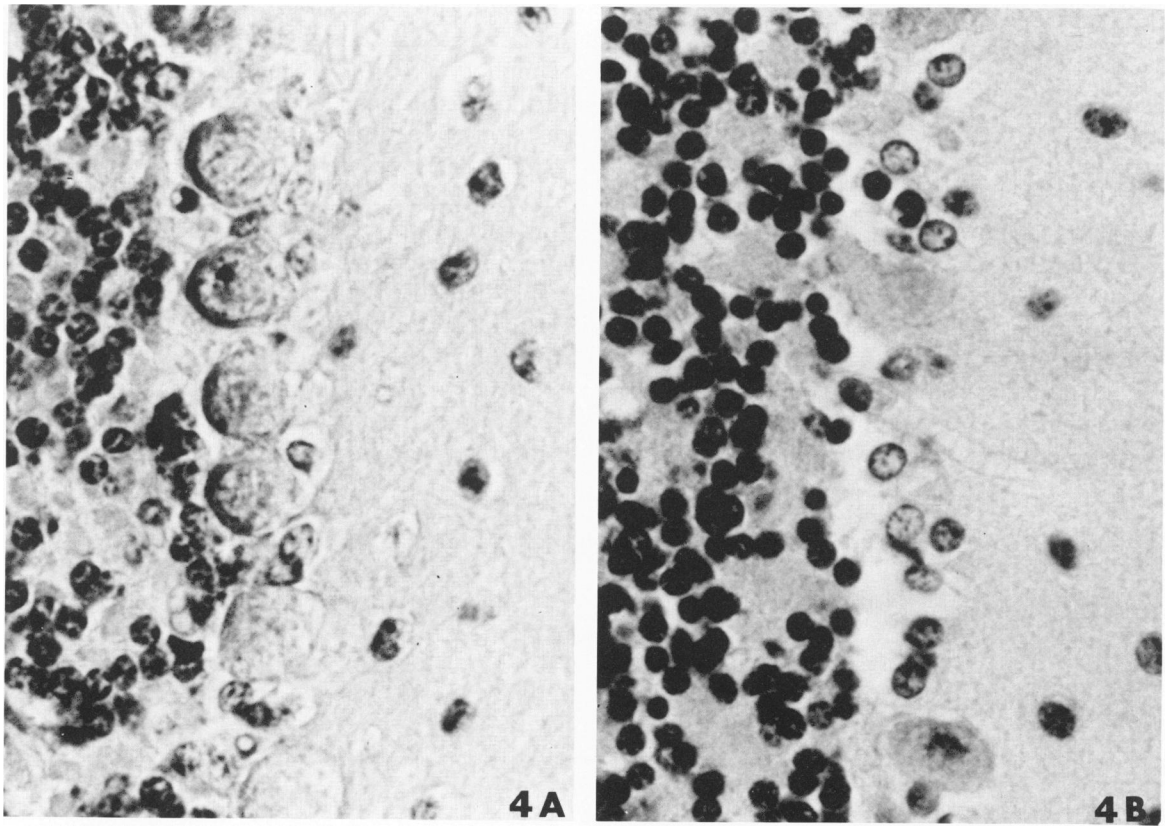

Figure 4—Focal dropout of Purkinje cells and Bergmann astrocyte proliferation in the cerebellum 2 days after a single injection of 10 μg/g DCF (**B**), as compared with a normal control (**A**). (×550)

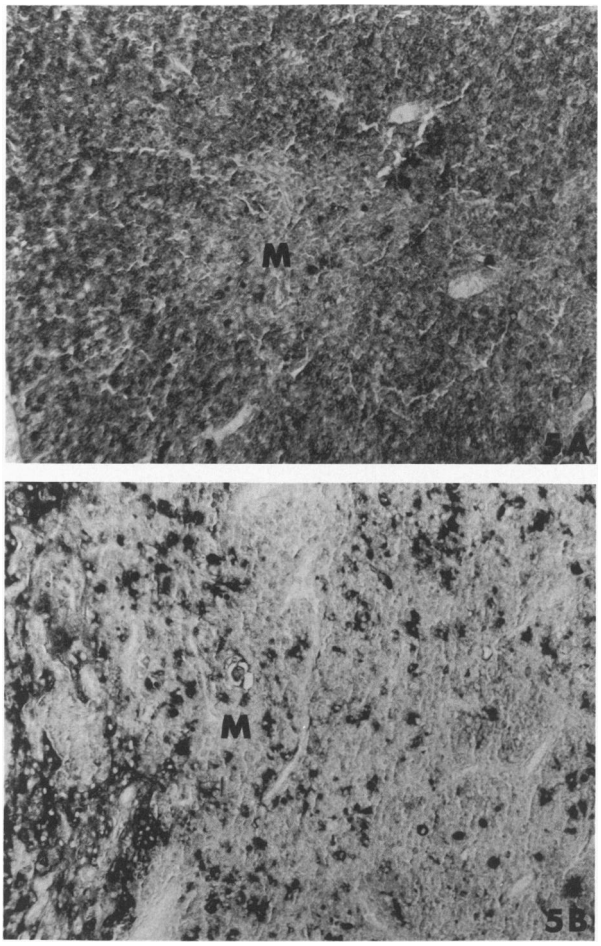

Figure 5—Normal thymus stained with PNA-peroxidase (**A**), as compared with a thymus taken 2 days after a single injection of 10 μg/g DCF. Note the specific cellular depletion of the cortex. *M*, medulla. (× 100)

sitivity of PNA-positive, immature thymocytes. Within days, the adrenal glands showed damage to the cortex at a low dose of DCF (10 μg/g) and additional damage to the medulla at a high dose of DCF (100 μg/g). It was only at the high dose of DCF that mice showed symptoms of an Addisonian-like crisis, including sluggish responses, tremors, and hypothermia.

The thymic changes would have been consistent with the effects of a sudden release of corticosteroids from the damaged adrenals. However, similar effects of DCF on thymocyte subpopulations in normal controls, as well as in adrenalectomized mice, proved that the relatively greater decrease in PNA-positive versus PNA-negative thymocytes observed was not due solely to release of lymphocytolytic steroids by the damaged adrenal glands, but was a direct consequence of DCF treatment.

The lymphoid depletion of the spleen and lymph nodes did not show histologic evidence of preferential T-cell versus B-cell sensitivity in contrast to the reports in the literature.[4,6] This difference may be related to the use of a higher dose of DCF and a single injection schedule in the present study as compared with the continuous infusion during several days of a low dose of DCF used by others.[4,6] As expected, lymphoid depletion in the spleen and lymph nodes was more severe after a high dose than after a low dose of DCF.

The degree of absolute lymphopenia in the peripheral blood remained the same after either the low or the high dose of DCF. Paradoxically, there was a marked myelocytosis (leukemoid reaction) in the peripheral blood after the high dose only. The maintenance of the peripheral blood lymphocyte count in the face of marked lymphoid depletion of the spleen and lymph nodes at the high dose suggests either a compensatory maintenance in circulatory lymphocyte pools or else a greater resistance of the remaining circulating lymphocytes to DCF. The low dose of DCF resulted in increased extramedullary hematopoiesis in the spleen, as shown by a doubling in spleen weight and histologically, whereas the high dose of DCF caused an excess of myeloid cells to appear in the peripheral blood while the spleen was depleted of cells.

Severe hemolysis and resulting anemia, greater after a high dose than after a low dose of DCF, may be explained on the basis of three possible pathogenetic mechanisms: 1) alterations in erythrocyte nucleotide pools, 2) a direct lytic effect of steroids released from damaged adrenal glands, and 3) oxidative damage to erythrocyte membranes due to release of xanthine oxi-

Table 3—Percent PNA-FITC-Positive Thymocytes in DCF-Treated Mice*

Mice	Method of Analysis	No DCF	Days After DCF†			
			+1	+2	+3	+4
Normal AKR/J	Cytofluorograph‡	83.6 ± 3.2	72.2 ± 4.3	52.3 ± 12.2	57.4 ± 9.3	69.0 ± 6.9
	IF microscopy§	76.8 ± 8.5	62.8 ± 6.4	37.0 ± 12.3	63.3 ± 15.5	69.4 ± 13.1
Adrenalectomized AKR/J	IF microscopy§	76;77	ND	ND	38;42	ND

* Mean ± SD; n = 5 mice per value, except adrenalectomized mice, which are listed as individual values.
† A single injection of 10 μg/g DCF was administered on Day 0.
‡ Cytofluorograf gated to remove RBCs; 10,000 cells/analysis.
§ Cells counted under a Leitz immunofluorescence (IF) microscope.

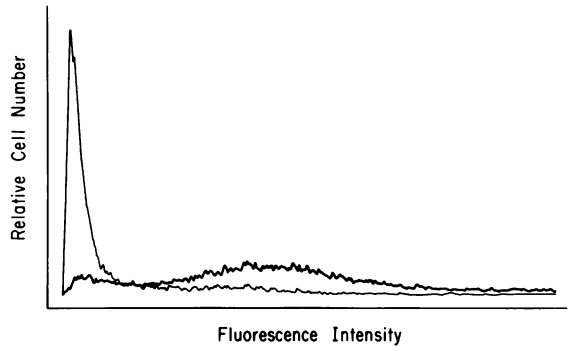

Figure 6—PNA-FITC-stained thymocytes from a normal mouse (*thick line*) and from a mouse killed 5 days after a single injection of 40 μg/g DCF (*thin line*). The percent of PNA-positive cells in the DCF-treated mouse was 15%, as compared with 85% in the control. This was a typical result, from an experiment in which 5 pairs of mice were compared.

dase and uricase from necrotic hepatocytes into serum. Alterations in erythrocyte nucleotide pools associated with hemolysis have been described in both DCF-treated mice[7] and in DCF-treated humans.[13] In addition, similar erythrocyte nucleotide pool changes have been reported in individuals with genetically determined ADA⁻-SCID,[14] eg, decreased adenosine triphosphate (ATP) and increased deoxyadenosine triphosphate (dATP) content. ADA⁻-SCID patients have not been reported to be anemic. Although increased hemolysis has been attributed to lowered ATP concentrations in transfused stored blood[15] and in hereditary abnormalities of erythrocyte metabolism, such as increased adenosine deaminase,[16] hemolysis in mice treated with DCF could not be correlated with changes in erythrocyte adenine nucleotide levels.[7] Hemolysis following DCF has been observed in BDF, BALB/c, C57BL/6,[8] and Swiss[7,8] mice. In contrast to the report of Spremulli et al,[8] we observed numerous Burr cells and fragmented cells following DCF-induced hemolysis. We speculate that the generation of oxidative-free radicals due to the release of xanthine oxidase and uricase from damaged liver could account for the difference in hemolysis between DCF-treated mice and humans versus humans with ADA⁻-SCID.

Acute tubular necrosis was only seen in the present study after a lethal single injection of 100 μg/g DCF, while Spremulli et al[8] used lower single doses or a low dose continuous infusion of DCF without observing hemoglobinuric nephropathy or death. Whether the acute tubular necrosis observed here was due to hemoglobinuria, ischemia, or direct toxicity remains to be determined. We were unable to induce any renal glomerular changes in adult mice detectable by light microscopy after as many as five single injections of 10 μg/g DCF during a 2-week period, nor was there any evidence of chondrocyte necrosis. We searched for these changes in mice treated with DCF because these organs showed characteristic pathologic changes in autopsy cases of ADA⁻-SCID.[9] The absence of these changes may be due to the fact that these studies were performed in adult mice, rather than in neonatal mice, whose tissues are still rapidly growing and may be more sensitive to ADA inhibition.

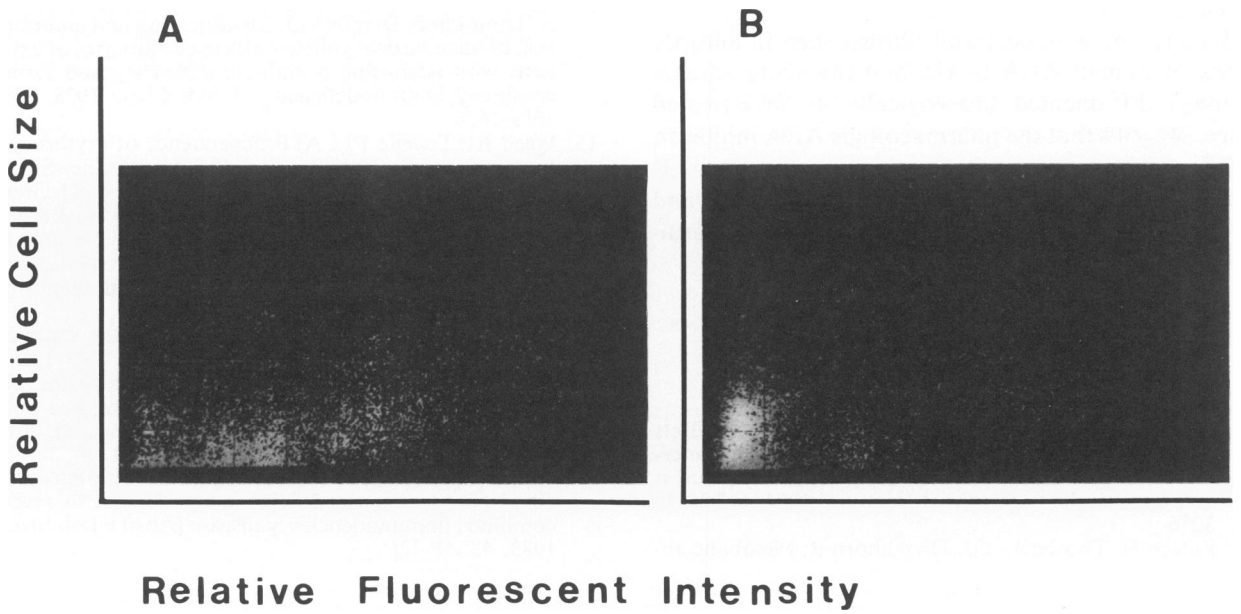

Figure 7—Ortho Cytofluorograf right-angle scatter (size) versus fluorescence dot plots showing a shift from normal, large, brightly fluorescent PNA-positive thymocytes (**A**) to small, dimly fluorescent PNA-positive thymocytes (**B**) 2 days after a single injection of 10 μg/g DCF (10,000 cells per analysis). These are typical dot plots for a pair of mice from experiments employing 5 mice in each group. These findings were highly reproducible, as can be seen in Table 3, line 1.

The Bergmann astrocyte proliferation and focal Purkinje-cell dropout seen in the cerebellum of mice after treatment with DCF is of possible interest in view of reported neurologic abnormalities in ADA⁻-SCID (reviewed in Hirschhorn[18]), as well as in patients treated with DCF (reviewed in Hirschhorn and Ratech[10]). We have also seen focal Purkinje-cell dropout in a single autopsy case of ADA⁻-SCID.[19] However, the relationship between these cerebellar changes in the mouse and DCF treatment is not proven, because similar alterations are readily obtained by a variety of other nonspecific stresses such as severe hypoxia consequent to marked hemolytic anemia.

These changes in DCF-treated mice have several implications for the pathophysiology of human ADA⁻-SCID, as well as for the use of DCF in the therapy of lymphoid malignancies. Of particular interest in this respect is the marked adrenal damage seen in the DCF-treated mice even at the lower dose level. A recent review of 8 autopsy cases of ADA⁻-SCID[9] showed fibrosis of the adrenal cortex in 6 cases. Acute adrenal damage in DCF-treated mice, therefore, supports the hypothesis that the changes seen in the human disease are related to metabolic consequences of the enzymatic deficiency of ADA rather than to a viral infection or other causes. Similarly, the preferential sensitivity of PNA-positive, immature thymocytes in mice, shown here to be due to a direct effect of DCF, suggests that a major defect in lymphoid development in patients with ADA⁻-SCID may occur at an early step during intrathymic differentiation, rather than at the stem-cell level.

Finally, the adrenal gland fibrosis seen in autopsy cases of human ADA⁻-SCID, and the acute adrenal damage documented histologically in DCF-treated mice, suggests that the pharmacologic ADA inhibitor, DCF, may have unsuspected toxicity for this organ. It may be speculated that carcinoma of the adrenal gland or neoplasms of neural crest derivation could be sensitive to chemotherapy by ADA inhibitors.

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