Enhanced Antibody Response in the Presence of Partial Adenosine Deaminase Inhibition (43051)

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Abstract. **The enzyme adenosine deaminase (ADA) catalyzes the conversion of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively. In the absence of ADA activity, 2′-deoxyadenosine is phosphorylated to deoxyadenosine triphosphate. This study concerned the effects of the ADA inhibitor 2′-deoxycoformycin on the murine *in vitro* immune response to sheep red blood cells (Mishell-Dutton cultures). In the presence of 10^{-7} *M* 2′-deoxycoformycin or 1 m*M* 2′-deoxyadenosine, there was a significant increase in the plaque-forming cell response when calculated as plaques per 10^6 viable cells recovered. Cultures containing 10^{-7} *M* 2′-deoxycoformycin retained approximately 10% of residual ADA activity of control cultures. Partial ADA deficiency was not preferentially toxic for cells capable of suppressing plaque cell generation. However, there was a decrease of recovered viable cells in all ADA-deficient cultures. There was no change in the percentage of recovered cells which were L3T4$^+$ or Lyt 2$^+$. A significant decrease was observed in a population of cells expressing surface immunoglobulins. The number of plaque-forming cells/10^3 recovered B cells increased significantly. We conclude that partial ADA deficiency results in selective toxicity to a population of non-antigen-specific B cells. Further studies with antigen-specific cells are necessary to determine the possible mechanism(s) by which cellular activation may prevent susceptibility to the toxic effects of ADA deficiency.** [P.S.E.B.M. 1990, Vol 194]

Adenosine deaminase (ADA), an enzyme in the purine salvage pathway, catalyzes the conversion of adenosine and deoxyadenosine (dado) to inosine and deoxyinosine, respectively. Children inherently devoid of ADA activity display a form of severe combined immunodeficiency disease (1). In the absence of ADA activity, dado is phosphorylated to deoxyadenosine triphosphate (dATP) (2, 3). The accumulated dATP inhibits ribonucleotide reductase activity and prevents DNA replication which is necessary for the cellular proliferation essential to the immune response. dATP also inhibits the repair of DNA strand breaks in both normal and damaged lymphocytes (4, 5). Accumulated dado prevents normal intracellular methylation reactions by inhibition of the enzyme *S*-adenosylhomocysteine hydrolase (6, 7). Individuals lacking ADA activity lack both B and T cells. Therefore, the observation that the plaque-forming cell (PFC) response in Mishell-Dutton cultures is increased in the presence of adenosine and/or coformycin was unexpected (8). Seegmiller *et al.* (8) concluded that the change in the PFC response occurred because T cells were more sensitive than B cells to the cytotoxic effects of ADA deficiency.

Nicholson *et al.* (9) introduced the use of Mishell-Dutton cultures for the *in vitro* study of ADA deficiency using the ADA-specific inhibitor 2′-deoxycoformycin (dCF). An increased number of PFC were recovered from Mishell-Dutton cultures incubated with dCF over a narrow concentration range. They also reported an increase in PFC/culture even though there was a concomitant decrease of viable cell recovery from cultures incubated with dCF in most experinents. All their data are expressed on the basis of PFC/10^6 viable cells recovered. However, they did not determine whether there was any residual ADA activity in the presence of dCF. The increase in the PFC response was attributed to either increased T helper cell activity, decreased T suppressor cell number or function, a selective toxicity of antigen-nonspecific B cells, or a combination of the three factors. They attempted to identify T and B cells

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by immunofluorescent methods after culture with dCF. However, their results were inconclusive because the large number of dead cells made quantitation very difficult. They proposed, without offering any direct proof, that the increase in the PFC response in the presence of dCF was due to a selective toxicity of dCF for a subset of T cells whose normal function was to dampen or suppress the PFC response.

The present study was undertaken to expand on the work of Nicholson *et al.* (9). Experiments were conducted with the ADA inhibitor dCF to determine the mechanism of the increase in the PFC response. The study was designed to determine (i) the extent of ADA inhibition in Mishell-Dutton cultures incubated with 10^{-7} *M* dCF; (ii) the effect of 10^{-7} *M* dCF and/or 1 m*M* dado on T and B cells; and (iii) whether accumulation of intracellular dATP was responsible for the change in the PFC response. The data presented provide evidence to support the hypothesis that inhibition of ADA by the ADA-specific inhibitor dCF results in a differential cytotoxicity for the nonactivated B cells. Selective sparing of antigen-stimulated B cells gives rise to an apparent increase in the *in vitro* immune response.

Materials and Methods

Cell Preparation and Culture. Female BDF_1 mice, purchased from Charles River Labs, Inc. (Wilmington, MA), were housed in a laminar flow bench and provided with food and water *ad libitum*. Mice were sacrificed by cervical dislocation at the age of 14–34 weeks. Spleens were removed aseptically into Hanks' balanced salt solution. Spleens were disrupted with frosted glass slides and filtered through Nitex monofilament mesh (Tetko, Inc., Elmsford, NY). Cell concentrations were determined on a Coulter Counter model ZBI (Coulter Electronics, Hialeah, FL). Cells were cultured according to the method of Mishell and Dutton (10). Cells were cultured at 8×10^6 cells/ml in Eagle's minimum essential medium for suspension culture (Whitaker/M. A. Bioproducts, Walkersville, MD) supplemented with 10% fetal calf serum (K. C. Biological, Lenexa, KS), nonessential amino acids, 1 m*M* sodium pyruvate, 2 m*M* L-glutamine, 2×10^{-5} *M* 2-mercaptoethanol, 50 units/ml penicillin, 50 μg/ml streptomycin, and 25 m*M* Hepes buffer. Sheep red blood cells (SRBC) (Hazleton Dutchland, Denver, PA and Crane Laboratories, Syracuse, NY), resuspended to 1% in Hanks' balanced salt solution, were added as the antigen. Appropriate cultures were treated with 2′-deoxyadenosine (Sigma Chemical Co., St. Louis, MO) and/or 2′-deoxycoformycin (a gift from Warner-Lambert, Ann Arbor, MI). Cultures were maintained in an atmosphere of 10% CO_2, 7% O_2, and 83% N_2 at 37°C for up to 5 days.

Plaque-Forming Cell Assay. Cells were recovered from culture at the end of 5 days and assayed for anti-SRBC PFC according to the Cunningham and Szenberg modification of Jerne's hemolytic plaque assay (11). Cultures were assayed in triplicate. Results were expressed as PFC/10^6 viable cells recovered or PFC/culture. Cell viabilities were determined by staining with 2 μg/ml propidium iodide (Sigma Chemical Co.).

Suppressor Cell Assays. Mishell-Dutton cultures were prepared as described above. Cultures were maintained for 3 days. Aliquots of 1×10^5 cells were added to freshly established Mishell-Dutton cultures which were maintained for 5 additional days (12). PFC were enumerated as described above.

ADA Assay. Splenocytes (1.5×10^7 cells) were harvested from culture and washed three times in phosphate-buffered saline. The cells were resuspended in 0.05 *M* $NaHPO_4$, pH 7.5 (13), and lysed by freezing at −70°C and thawing at 37°C five times. Cell membranes were removed by centrifugation at 20,000*g* for 30 min at 4°C. The cell extract was incubated with 1.5 m*M* adenosine in 0.05 *M* $NaHPO_4$ (pH 7.5) for 15 min at 37°C. Background concentrations of adenosine and inosine were determined by incubation of cell extracts without adenosine. The reaction was terminated by placement at 4°C. An aliquot was removed for protein determination by a modification of the Bradford method (14). The remaining reaction mixture was deproteinated with perchloric acid and neutralized with KOH. Extracts were stored at −70°C until analysis by high-performance liquid chromatography. Nucleosides were analyzed with a Zorbax TMS column (DuPont, Wilmington, DE) attached to a Spectra Physics model 8700 gradient system (Spectra Physics, San Jose, CA). Buffer A was 20 m*M* KH_2PO_4 (pH 4.45); Buffer B consisted of a 60:40 mixture of methanol and water (15). A linear gradient increasing to 25% B in 10 min was achieved with a flow rate of 2 ml/min. The unknown nucleosides were identified and quantitated by comparison to retention times and peak heights of known standards at the wave length of 254 nm as detected by a Spectra Physics 8440 detector and 4270 computing integrator. Specific ADA activity is expressed as nmol inosine formed/min/mg protein.

Immunofluorescent Staining of Cells. Hybridomas ATCC TIB 105 and 207 (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 (Whitaker/M. A. Bioproducts) supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, and 50 μg/ml gentamicin. TIB 105 (53–6.72) is a rat-mouse hybridoma which secretes rat IgG_{2a} antibodies that react with the lymphocyte differentiation antigen Lyt 2 (all alleles) (16). TIB 207 (GK 1.5), a rat-mouse hybridoma, produces rat IgG_{2b} antibodies that react with the T cell surface antigen L3T4 expressed on the helper/inducer subset of murine T cells (17). Culture supernatants containing anti-L3T4 and anti-Lyt 2 antibodies were labeled with fluorescein isothiocyanate (FITC) (Sigma Chemical Co.) (18). B cells were labeled with FITC-

conjugated goat anti-mouse IgG (heavy and light chain specific) (Cappel, West Chester, PA).

Cells (5×10^6) were incubated in fetal calf serum for 10 min at 4°C, and subsequently incubated with antibody diluted in phosphate-buffered saline with 0.1% sodium azide (phosphate-bufferd saline/azide) for 30 min at 4°C. Red blood cells were removed by the addition of tris-buffered ammonium chloride for 10 min at room temperature. Cells were washed twice and resuspended in phosphate-bufferd saline/azide, and held on ice. A final concentration of 2 μg/ml propidium iodide was added to the cells 15 min prior to analysis by flow cytometry.

Cytofluorographic Analysis. Lymphocyte suspensions were analyzed by flow cytometry with a Cytofluorograf IIS (Ortho Diagnostics Systems, Inc., Westwood, MA) with argon laser at 488 nm. Data were collected for analysis on a model 2140 computer interface. Samples were analyzed based on forward (size) and right angle (granularity) scatter characteristics. A gate was established around the lymphocyte population and the green (FITC) fluorescence of cells within the gated region determined. The percentage of positive stained cells was determined by comparison to an unstained control. Viable lymphoid cells were distinguished from nonviable cells by their failure to stain with the vital dye propidium iodide.

Statistical Analysis. The data were tested by one-way analysis of variance. If the outcome of the analysis of variance was significant (i.e., the P value of the F ratio was <0.05), an honestly significant difference was performed.

Results

Effects of the addition of 10^{-7} M dCF or 10^{-8} M dCF to Mishell-Dutton cultures are shown in Figure 1. There was decreased recovery of viable cells in cultures incubated with dCF as compared with control cultures (Fig. 1A). There was also a decrease in the absolute number of PFC/culture obtained from cultures incubated with dCF (Fig. 1C). However, a significant increase in PFC/10^6 viable cells recovered was observed in cultures incubated with 10^{-7} M dCF as compared with control cultures (Fig. 1B).

A series of experiments were undertaken to determine whether the addition of dado to Mishell-Dutton cultures would result in a similar increase in the PFC response. Figure 2A shows decreased viable cell recovery in cultures containing 1 mM dado. There was also a decrease in the number of PFC/culture (Fig. 2C). Similar to results obtained with dCF, there were significant increases in the number of PFC/10^6 viable cells recovered at 1 mM dado (Fig. 2B).

To determine whether the concentration of dCF present in the Mishell-Dutton cultures was sufficient to completely inhibit the ADA activity in the cells, dilutions of calf intestinal ADA were incubated with varying concentrations of dCF. It was determined that the dCF concentration of 10^{-10} M was sufficient to inhibit almost 10-fold the amount of ADA calculated to be present in the cells in Mishell-Dutton cultures (data not shown). The effect of dCF was measured over the course of the first 24 hr of culture (Table I). It was found that ADA activity was never completely inhibited. ADA activity decreased in control cultures over time and at

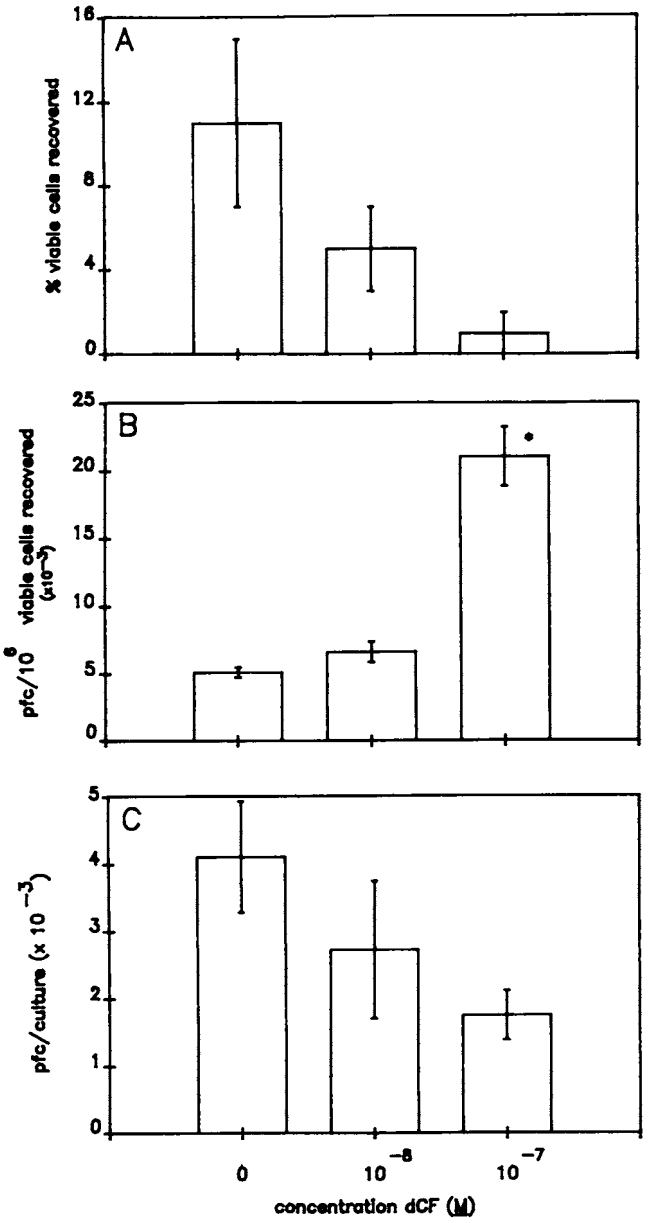

Figure 1. The effect of dCF on the PFC response in Mishell-Dutton cultures. Freshly explanted murine splenocytes (8×10^6) were cultured in the presence of SRBC and 10^{-7} or 10^{-8} M dCF. Cells were harvested on Day 5. (A) The percentage of viable cells recovered as determined by propidium iodide exclusion. PFC response expressed in terms of PFC/10^6 viable cells recovered (B) and PFC/culture (C). Results are expressed as mean and standard error obtained in three experiments. *Data are significantly different ($P < 0.01$) from the control.

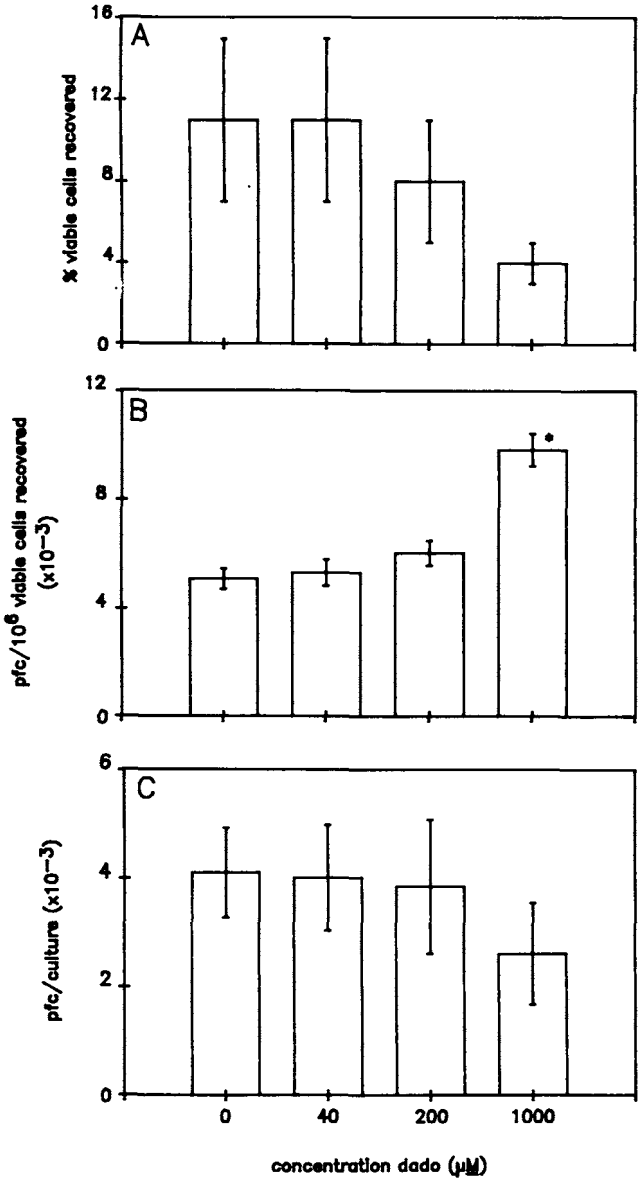

Figure 2. The effect of dado on the PFC response in Mishell-Dutton cultures. Freshly explanted murine splenocytes (8×10^6) were cultured in the presence of SRBC and increasing concentrations of dado. Cells were harvested on Day 5. (A) The percentage of viable cells recovered as determined by propidium iodide exclusion. PFC response was expressed in terms of PFC/10^6 viable cells recovered (B) and PFC/culture (C). Results are expressed as mean and standard error obtained in three experiments. Data are significantly different ($P < 0.05$) from the control.

24 hr only 46% of initial activity could be detected. Significant inhibition of ADA activity in dCF cultures did not occur until 4 hr of culture. Inhibition of ADA was approximately 90% of residual control activity at 24 hr in culture.

Experiments were undertaken to determine whether the increase in the PFC response was due to a selective effect of dCF on suppressor cells. Splenocytes harvested from Mishell-Dutton cultures, when added to fresh Mishell-Dutton cultures, inhibited the PFC

Table I. Inhibition of ADA activity by 10^{-7} *M* dCF during the first 24 hr of Mishell-Dutton Cultures[a]

Time (hr)	Specific ADA activity (nmol inosine formed/min/mg protein)		% residual ADA activity (inhibited/control × 100)
	Control cultures	Inhibited cultures	
0	81.6 ± 5.8	83.4 ± 14.2	101 ± 14
2	49.8 ± 6.7	49.5 ± 15.1	95 ± 17
4	51.7 ± 8.3	28.5 ± 4.7	55 ± 5[b]
6	39.4 ± 5.5	15.7 ± 2.2	40 ± 1[b]
8	45.7 ± 7.6	13.8 ± 3.7	29 ± 3[b]
24	37.7 ± 7.6	3.2 ± 1.3	8 ± 2[c]

[a] Data are expressed as the mean ± SE of three experiments.
[b] Data are significantly different ($P < 0.05$) from those of the control.
[c] Data are significantly different ($P < 0.01$) from those of the control.

Table II. Effect of dCF on the Suppression of the PFC Response in Mishell-Dutton Cultures by the Addition of Splenocytes from Previous Mishell-Dutton Cultures[a]

Addition of cultured cells	dCF of cultured cells (*M*)	% viable cells recovered	PFC/10^6 viable cells recovered
–	—	15 ± 2	1540 ± 320
+	—	17 ± 2	158 ± 67[b]
+	10^{-7}	17 ± 2	204 ± 92[b]
+	10^{-8}	18 ± 2	112 ± 38[b]

[a] Results are expressed as mean ± SE of five experiments.
[b] Data are significantly different ($P < 0.01$) from those of the control.

response of the second culture (Table II). Addition of splenocytes from Mishell-Dutton cultures incubated in the presence of dCF showed comparable suppression of the PFC response of fresh Mishell-Dutton cultures. Therefore, it did not appear that dCF was selectively cytotoxic for suppressor cells.

T cells recovered from Mishell-Dutton cultures were labeled with FITC-conjugated anti-Lyt 2 and FITC-conjugated anti-L3T4 to determine the effects of dado and/or dCF on subpopulations of T cells (Table III). Although there was a significant decrease in viable cell recovery in cultures containing 1 m*M* dado and/or 10^{-10} *M* dCF, there was no significant change in the percentage of cells which were L3T4$^+$ or Lyt 2$^+$ or the ratio of L3T4 to Lyt 2$^+$ cells.

B cells recovered from Mishell-Dutton cultures were identified by labeling with FITC-conjugated anti-mouse IgG (heavy and light chains specific). Table IV presents the effects of dado and/or dCF on the B cell populations in Mishell-Dutton cultures. A significant decrease was observed in the percentage of viable IgG$^+$ cells recovered from cultures incubated with 1 m*M* dado (Table IVB) but not from cultures incubated with 10^{-7} *M* dCF (Table IVA). There was a decrease in the

Table III. Effects of dado and dCF on Subpopulations of T Cells in Mishell-Dutton Cultures as Determined by Flow Cytometry

Days in culture	dCF (*M*)	Dado (*mM*)	% viable cells recovered[a]	% L3T4$^+$	% Lyt 2$^+$	% L3T4$^+$/% Lyt 2$^+$
0[b]	—	—	—	15.7 ± 1.9	9.5 ± 0.5	1.6 ± 0.1
5[b]	—	—	14 ± 3	25.2 ± 3.6	16.5 ± 1.2	1.5 ± 0.2
5[b]	—	1	5 ± 1[c]	32.2 ± 2.4	17.9 ± 1.6	1.8 ± 0.2
5[b]	10^{-7}	—	3 ± 1[c]	25.0 ± 2.2	19.2 ± 2.8	1.3 ± 0.2
5[d]	10^{-7}	1	<1[c]	34.0 ± 16.0	21.3 ± 0.5	1.8 ± 0.9

[a] Viable cells excluded propidium iodide.
[b] Results are expressed as mean ± SE obtained in seven experiments.
[c] Data are significantly different ($P < 0.05$) from those of the control.
[d] Results are expressed as mean ± SE obtained in two experiments.

PFC/culture both in the presence of 10^{-7} *M* dCF (Table IVA) and in the presence of 1 m*M* dado (Table IB). There was a significant increase in the number of PFC/10^3 viable B cells recovered from cultures incubated with 10^{-7} *M* dCF (Table IVA) and from cultures incubated with 1 m*M* dado (Table IVB). Similar results were obtained when cells were labeled with either FITC-conjugated anti-mouse IgG (γ chain specific) or FITC-conjugated anti-mouse IgM (μ chain specific) (data not shown).

Discussion

The first goal of this study was to determine whether an increase in PFC response could be detected in cultures incubated with dCF. We found a comparable increase in PFC/10^6 viable cells recovered. However, incubation with dCF often resulted in a decrease in PFC/culture. In order to test the hypothesis that the cytotoxic effects of dCF were on suppressor cells, we conducted suppressor cell assays with cells from Mishell-Dutton cultures that contained dCF. Ryoyama *et al.* (19) had reported that spleen cells harvested fron Mishell-Dutton cultures represented a suppressor population which, when added to additional Mishell-Dutton cultures, inhibited the PFC response of the second cultures. We confirmed that observation. Use of cells obtained from cultures containing dCF as the suppressor population resulted in suppression of the PFC response which was comparable to control cultures. Therefore, dCF did not appear to be selectively cytotoxic for the subset of T suppressor cells responsible for inhibition of PFC responses.

A series of experiments was undertaken to determine whether the incubation of cells in Mishell-Dutton cultures with dado would result in an increased PFC response as compared with control levels. Nicholson *et al.* (9) reported an increase in the PFC response of Mishell-Dutton cultures incubated with dado, but did not report the concentration range of dado that would yield the increase in the PFC response nor the magnitude of the response. In the present study, over the concentration range of 0–1 m*M* dado, cultures containing 1 m*M* dado had the greatest increase in PFC/10^6 viable cells as compared with control levels. There was no effect on the PFC/culture response.

An important consideration for interpretation of results was whether dCF inhibited ADA activity in cultures. Experiments were undertaken to determine the extent of ADA inhibition by 10^{-7} *M* dCF during the first 24 hr in culture. It is during the first 24 hr that the interactions of the antigen-presenting macrophages and B cells with T helper cells initiate an antigen-specific immune response. Experiments with purified ADA had shown that there was sufficient dCF in the Mishell-

Table IV. Effects of dCF or dado on SRBC-specific B cells in Mishell-Dutton Cultures as Determined by Flow Cytometry[a]

	Dado (μM)	dCF (*M*)	PFC/culture	% IgG$^+$ viable recovered cells	PFC/10^3 viable B cells recovered
A.	—	—	4111 ± 825	60.3 ± 1.7	8.5 ± 1.6
	—	10^{-8}	2729 ± 1020	66.0 ± 1.5	10.0 ± 0.6
	—	10^{-7}	1756 ± 368	55.3 ± 5.3	42.8 ± 8.1[b]
B.	40	—	4019 ± 973	59.0 ± 2.1	9.0 ± 1.2
	200	—	3858 ± 1235	62.3 ± 3.1	12.0 ± 2.0
	1000	—	2621 ± 937	40.8 ± 0.9[b]	18.9 ± 1.5[c]

[a] Results are expressed as mean ± SE of three experiments.
[b] Data are significantly different ($P < 0.01$) from those of the control.
[c] Data are significantly different ($P < 0.05$) from those of the control.

Dutton cultures to completely inhibit the calculated quantity of ADA present in the splenocytes. However, residual ADA activity was present in cultures at a level of approximately 30% that of control cultures after 8 hr of culture. The maximum inhibition of ADA activity (approximately 90%) was observed after 24 hr in culture. Siaw and Coleman (20) reported a similar observation in T lymphoblastoid cells incubated with 10^{-6} or 10^{-7} *M* dCF. In that study the intracellular concentration of dCF was determined to be approximately one-half the concentration of dCF in the culture medium. Maximum intracellular concentration was achieved by 10 hr in culture. The finding that ADA was not completely inhibited in the presence of 10^{-7} *M* dCF made it evident that Mishell-Dutton cultures incubated with 10^{-7} *M* dCF did not provide an adequate model for patients with severe combined immunodeficiency disease. Rather, these cultures serve as a model for people who are partially deficient in ADA, yet remain immunocompetent. This study provided a way of determining the fundamental difference in the effect of ADA deficiency on various lymphocyte populations.

In a series of experiments performed to assess the selective toxicity to populations of B and T cells, there was no significant difference in the percentage of cells which were $L3T4^+$ or Lyt 2^+ among viable cells from cultures containing dCF and/or dado, in spite of the reduced recovery of viable cells. Also, there was no significant difference in the ratio of $L3T4^+$ cells to Lyt 2^+ cells. Therefore, there was no change in the T helper or T suppressor population which could account for the change in the PFC response under the conditions of incomplete ADA deficiency.

B cells from Mishell-Dutton cultures incubated with dCF and/or dado were identified by labeling with FITC-conjugated anti-mouse IgG (heavy and light chain specific). There was a significant difference in viable cell recovery from cultures incubated with either 1 m*M* dado and/or 10^{-7} *M* dCF. A significant decrease was observed in the percentage of viable cells expressing surface immunoglobulin in cultures containing 1 m*M* dado. Similar decreases were found in B cell populations labeled with other anti-immunoglobulin antibodies. Thus, it appeared that the selective toxicity of dado and/or dCF was not on helper or suppressor T cells, but on a B cell population. The conclusion was drawn that the cytotoxic effect of partial ADA deficiency was on nonantigen-specific B cells, resulting in a relative increase in antigen-specific B cells giving rise to an apparent increase in the PFC response. This conclusion was strengthened by the determination that there was an increase in the number of PFC/10^3 viable B cells recovered. It appears that the activation of antigen specific B cells protected the cells from the cytotoxicity of ADA inhibition, and that cells which were not activated were more susceptible to ADA inhibition. It was not possible to detect the toxic metabolite dATP in any populations of cells recovered from Mishell-Dutton cultures with partial ADA inhibition (data not shown). Either dATP does not accumulate with partial ADA inhibition or the dATP isolation procedure removed the nonviable cell populations which were most likely to have intracellular dATP present. This procedural difficulty could be alleviated by studying the effect of partial ADA inhibition in antigen-specific T and B cell populations.

Partial ADA deficiency in Mishell-Dutton cultures resulted in an increase in the number of PFC/10^6 viable cells recovered. A suppressor assay revealed that partial ADA deficiency was not preferentially cytotoxic for suppressor cells. There was a significant decrease of recovered viable cells in all ADA-deficient cultures. Although there was no change in the percentage of $L3T4^+$ and Lyt 2^+ cells and no change in the ratio of $L3T4^+$ to Lyt 2^+ cells, there was a significant decrease in a population of cells expressing surface immunoglobulins. There was also a significant increase in the number of PFC/10^3 viable B cells recovered. It appeared that the selective toxicity of partial ADA deficiency was to a population of B cells which was nonantigen specific and that there is a selective toxicity for ADA deficiency among splenocytes. Further studies with antigen-specific B and T cells are necessary to determine the possible mechanism(s) by which cellular activation may prevent susceptibility to partial ADA deficiency, and to determine whether there is a fundamental difference in B and T cell activation which underlies the enhanced B cell susceptibility to the toxicity of ADA inhibition.

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