Modulation of the Antibody Response to Sheep Red Blood Cells in Normal and Immunodeficient XID Mice by *myo*-Inositol

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Abstract. **Over the dose ranges tested, dietary vitamin A and *myo*-inositol induce similar changes in murine fetal growth and development. Because vitamin A is also known to affect the immune response, studies were conducted to determine if dietary *myo*-inositol might have an effect on antibody production. It was found that *in vivo* in inbred mice *myo*-inositol (4 mg/g of diet) accelerated the rate of appearance of plaque-forming cells (PFC) in a primary response to sheep red blood cells (SRBC). *In vitro*, *myo*-inositol accelerated the rate of appearance of colonies of anti-SRBC PFC (foci) and significantly increased the number of PFC per colony, but did not affect the number of foci per culture noted at the end of the culture period. *myo*-inositol had no effect on the PFC IgM:IgG ratio following a single exposure to the agent, but exposure to *myo*-inositol *in vivo* and *in vitro* resulted in a decrease in the number of IgM PFC per focus in a primary response and IgM and IgG PFC per focus in a secondary response. Based on studies suggesting that *myo*-inositol or a phosphorylated metabolite might act downstream from Bruton's tyrosine kinase (Btk) in a signal transduction pathway in B cells, immunodeficient CBA/CaHN-XID/J mice were fed a standard diet or the same diet supplemented with 0.4% *myo*-inositol. Mice given the supplemented diet produced significantly more IgM anti-SRBC antibody than did XID mice given the control diet (4.3 ± 2.5 vs 1.7 ± 2.8, $1/\log_2$), and produced approximately the same amount as immunocompetent controls (2.9 ± 0.9). When rechallenged with SRBC, XID mice given supplemental inositol produced significantly more IgM antibody than did the XID and immunocompetent controls (3.6 ± 0.5 vs 1.8 ± 1.1 and 1.5 ± 0.7, respectively). Added dietary inositol did not have a significant effect on primary or secondary IgG responses to SRBC, which remained impaired. These results suggest that dietary *myo*-inositol or a derivative may be able to modulate B-cell IgM responses by interacting within the inositol second messenger system downstream from Bruton's tyrosine kinase.** [P.S.E.B.M. 1997, Vol 215]

Recent work from this laboratory has shown that 11-day-old mouse fetuses from mothers fed diets supplemented with vitamin A, 200 IU/day, or *myo*-inositol, 0.1% (w/w), are approximately 25% larger and have significantly advanced development of the eyes and hind limbs compared with fetuses from dams fed a standard mouse chow, Purina 5001 (1). At higher doses of vitamin A (400 and 500 IU/day) and *myo*-inositol (0.4%), growth and development was retarded in 11-day-old fetuses (unpublished data), and, at term, body weight was reduced in fetuses from dams bearing *d* alleles distal to Eα in the H-2 complex. The frequency of microphthalmia was increased in both treatment groups (2). Taken together, these observations show that, over the dose ranges tested, dietary vitamin A and *myo*-inositol induce similar changes in growth and development.

Because vitamin A status is also known to affect various aspects of the immune response (3), it was decided to determine what effect, if any, *myo*-inositol might have on antibody production. Therefore, studies were conducted *in vivo* and *in vitro* on the effects of *myo*-inositol on the pri-

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mary and secondary antibody response to sheep red blood cells (SRBC). In addition, based on the hypothesis that *myo*-inositol or a phosphorylated derivative might act downstream from Bruton's tyrosine kinase (Btk) in the B-cell signal transduction pathway (4–7), similar experiments were performed *in vivo* on Btk-deficient, immunodeficient XID mice.

Materials and Methods

Experimental Animals. Twelve- to twenty-week-old male and female B10.A(15R) and B10.A(18R) mice, and 12-week-old CBA/CaHN-XID/J males (Jackson Laboratory, Bar Harbor, ME) were used. The mice were housed four to five per cage in an AAALAC-approved facility and given food and water *ad libitum.*

Diets. The mice were fed standard mouse chow (Purina 5001) or the same diet supplemented with 0.4% (4 mg/g) *myo*-inositol (tissue culture grade; Sigma Chemical Co., St. Louis, MO). The *myo*-inositol was dissolved in distilled water, and the 5001 bisquits were soaked in the solution (400 mg/100 g Purina 5001) and then dried as previously described (1). The average mouse consumes approximately 5 g of food per day and, therefore, would ingest approximately 20 mg of added *myo*-inositol.

***In Vitro* Immune Response.** Spleens were removed aseptically from donor mice, and the cells were gently dissociated in Hanks' balanced salt solution, washed once, and suspended in culture medium at a concentration of $10–20 \times 10^6$ viable cells/ml. Viability was determined using the trypan blue exclusion method (8).

All cultures were done in triplicate, and replicate samples were generally within 5% of the mean. The culture medium was RPMI-1640 supplemented with L-glutamine, 10% heat-inactivated fetal calf serum, and penicillin and streptomycin (100 U and 100 μg/ml, respectively).

Primary responses to SRBC were elicited as described previously (8). Briefly, an underlay of washed SRBC (2%) in 1.5 ml of agar (0.25%) was placed in 35 × 10–mm tissue culture dishes (Falcon Plastics, Oxnard, CA), and 3 ml of spleen cell suspension was added after the agar had hardened. *myo*-inositol was added to the cultures at the indicated concentrations, and the cells were cultured at 37°C in 5% CO_2 in air for 1–3 days. Medium and cells were then carefully removed, and 1 ml of guinea pig complement (1:7, absorbed with SRBC, Sigma) was added to each dish. After 1.5 hr at 37°C and 2 hr at room temperature, the areas of focal hemolysis were counted.

Previous work (Tyan, unpublished data) indicates that each area of hemolysis found in the agar represents a cluster of specific antibody-forming cells (PFC; focus, analogous to a colony of bacteria). When the cells are carefully removed from the agar and plated on another SRBC-agar underlay to detect single PFC (analogous to a single bacterium from the colony), from 1 to 70 PFC were found for each area of hemolysis noted on the original plate. Each area of hemolysis is the result of the production of specific antibody because (i) it is complement dependent, (ii) cells cultured on agar without SRBC do not contain SRBC-PFC on transfer, and (iii) cells cultured on SRBC-agar do not contain PFC to horse RBC or vice versa.

PFC. Cells from the spleens of mice immunized with SRBC *in vivo* (0.2 ml 20% SRBC iv) and cells taken from culture dishes at the termination of the experiments were assayed for direct (IgM) and indirect (IgG) PFC by methods described previously (8). Briefly, cells were added to 0.5% agar containing 2% SRBC and incubated 1.5 hr at 37°C. Guinea pig complement (1:7) previously absorbed with SRBC was added to the plates, which were then incubated at 37°C for 1 hr, followed by 2 hr at room temperature, after which the number of direct (IgM) PFC were counted. Then, goat anti-mouse IgG (1:100, Sigma) was added. The plates were incubated 1 hr at 37°C, SRBC-absorbed complement was added, and incubation continued for 1 hr at 37°C and 2 hr at room temperature. Individual hemolytic areas were counted, and the number of indirect (IgG) PFC calculated (Total PFC – Direct PFC = Indirect IgG PFC).

Studies on XID Mice. Forty CBA/CaHN-XID/J male mice were maintained on a Purina 5001 diet. Seven days before being bled, 20 of the mice were started and continued on Purina 5001 supplemented with 0.4% *myo*-inositol. Two days later, all of the XID mice and 10 immunocompetent 12-week-old CBA/CaJ controls were given 0.2 ml 20% SRBC (BBL) iv: 5 days later, the mice were bled from the retroorbital plexus. Hemagglutination titers (doubling dilutions) were determined by standard methods (9) on untreated serum and after treatment with dithiothreitol (DTT, Sigma) (10). Assays were performed at least twice on all samples. IgM titer refers to titer of untreated serum minus titer of DTT-treated serum (IgG). Five mice from each of the groups described above were given a second iv injection of 0.2 ml 20% SRBC 1 week after being bled the first time. Five days later, they were bled, and hemagglutination titers were determined. Total spleen PFC were determined on XID mice from each diet group and on 10 control immunocompetent mice.

Statistical Analysis. Means were compared using the paired *t* test and the Mann-Whitney two-sample test (NCSS statistical program; NCSS, Kayville, UT). The most conservative *P* value (i.e., the least significant) is reported without correction for multiple determinations where both methods were used to compare the same data.

Results

Seven immunocompetent mice on the standard diet and an equal number on the same diet supplemented with 0.4% *myo*-inositol were given 0.2 ml 20% SRBC iv and serum obtained 5 days later. The anti-SRBC hemagglutination titers (doubling dilutions) were higher in the group given the added *myo*-inositol (10.95 ± 0.48 vs 9.85 ± 0.90, $P = 0.047$) due entirely to increased IgM antibody (data not shown). These mice were continued on the same diets and rechallenged with SRBC on three occasions; no subsequent

differences in hemagglutination titers were noted (data not shown).

Spleen cells from naïve mice (B10.A[15R] or B10.A[18R]) were cultured with SRBC in the presence or absence of *myo*-inositol (1.0, 4.0, or 8.0 mg/ml), and after 3 days the number of foci and PFC/focus were determined. *myo*-Inositol at the concentrations used had no effect on the number of foci generated, but at a concentration of 4.0 mg/ml there were three times as many PFC/focus as in the control cultures (Table I).

Spleen cells from naïve mice were cultured with SRBC in the presence or absence of *myo*-inositol, 4.0 mg/ml, and number of foci generated was determined on Days 1, 2, and 3. It was found that the cultures with added inositol had significantly more foci than the controls on Day 1 but not thereafter (Table II).

Mice on the standard diet or the inositol-supplemented diet were given 0.2 ml 20% SRBC iv and their spleens were assayed for PFC on Days 3 and 5. Mice on the inositol-supplemented diet had twice the number of direct PFC per spleen as controls on Day 3, but no difference was noted on Day 5 (Table III).

Naïve mice were maintained on the control diet or placed on the inositol-supplemented diet 4 days before their spleen cells were cultured with SRBC with or without added *myo*-inositol. The number of foci per culture was not affected by exposure of the spleen cells to *myo*-inositol *in vivo* and/or *in vitro;* however, direct PFC/focus were increased when the spleen cells were exposed to inositol either *in vivo* or *in vitro,* and they were markedly decreased when exposed both *in vivo* and *in vitro* (Table IV). Similarly, the ratio of indirect (IgG) to direct (IgM) PFC was unaffected by a single *in vivo* or *in vitro* treatment with inositol, but IgG PFC were severely decreased on double exposure when measured at the end of secondary response (data not shown).

When naïve immunodeficient XID mice were fed the standard mouse chow or the same diet supplemented with 0.4% inositol and immunized with SRBC, it was found that the mice given the inositol-supplemented diet produced significantly more anti-SRBC hemagglutinating antibody than the mice on the control diet, but considerably less than immunocompetent controls fed the standard diet (Fig. 1). The increased antibody noted in the experimental group was largely, if not entirely, IgM.

At the time the mice were bled to assay the primary antibody response to SRBC, the majority of them were sacrificed to determine the number of anti-SRBC PFC in their spleens. It was found that the supplemented diet had no significant effect on the number of IgM or IgG PFC found

Table I. Effect of *myo*-Inositol on the Generation of Anti-SRBC Foci and PFC per Focus in 3-Day Cultures of Spleen Cells from Naïve Donors

Assay	*myo*-Inositol (mg/ml)		
	1.0	4.0	8.0
Foci[a]	1.32 ± 0.056 (8)	1.10 ± 0.31 (19)	0.99 ± 0.84 (6)
PFC/focus[b]	1.56 ± 1.22 (6)	3.24 ± 2.90[c] (10)	1.65 ± 1.80 (5)

Note. Values are expressed as mean ± S.D. Numbers in parentheses refer to number of experiments.
[a] The data have been normalized: foci in cultures with added inositol/foci in cultures without.
[b] PFC per focus in cultures with added inositol/PFC per focus in cultures without.
[c] $P < 0.01$, paired *t* test.

Table II. Effect of *myo*-Inositol on the *in Vitro* Primary Response to SRBC on Days 1, 2, and 3

Inositol (4 mg/ml)	Foci/culture		
	Day 1	Day 2	Day 3
No	1.88 ± 1.06[a] (6)	25.8 ± 15.3 (6)	24.0 ± 9.4 (6)
Yes	5.33 ± 2.56[a]	22.6 ± 16.6	23.1 ± 14.1

Note. Values are expressed as mean ± SD. Numbers in parentheses refer to number of experiments.
[a] $P = 0.004$, paired *t* test.

Table III. Effect of Added Dietary *myo*-Inositol (4 mg/g) on Spleen PFC 3 and 5 Days after a Primary Challenge with SRBC

Inositol (4 mg/g)	Direct PFC/spleen ($\times 10^3$)	
	Day 3	Day 5
No	0.93 ± 0.45[a] (7)	39.9 ± 19.8 (6)
Yes	2.26 ± 1.08[a]	37.2 ± 24.9

Note. Values are expressed as mean ± SD. Numbers in parentheses refer to number of experiments. Mice were started on the supplemented diet 2 days before challenge.
[a] $P < 0.01$, paired *t* test.

Table IV. Effects of *myo*-Inositol (4mg/g) on the Primary *in Vitro* Immune Response to SRBC when Added to the Diet of the Donor 4 Days before Sacrifice and/or to the Cultures

myo-Inositol in cultures (4 mg/ml)	Control diet		*myo*-Inositol diet (4 mg/g)	
	Direct foci/ spleen[a]	Direct PFC/ focus[b]	Direct foci/ spleen	Direct foci/ focus
No	26.2	0.97[c,d]	28.4	2.01[d,e]
Yes	28.5	2.52[c,f]	29.4	0.49[e,f]
SE diff.	1.94	1.00	8.32	0.47
n[g]	19	10	5	5

[a] Mean ($\times 10^3$). Groups are compared using the paired *t* and Mann-Whitney tests.
[b] Cells recovered from cultures on Day 3.
[c] $P = 0.028$.
[d] $P = 0.037$.
[e] $P = 0.024$
[f] $P = 0.034$.
[g] Number of experiments.

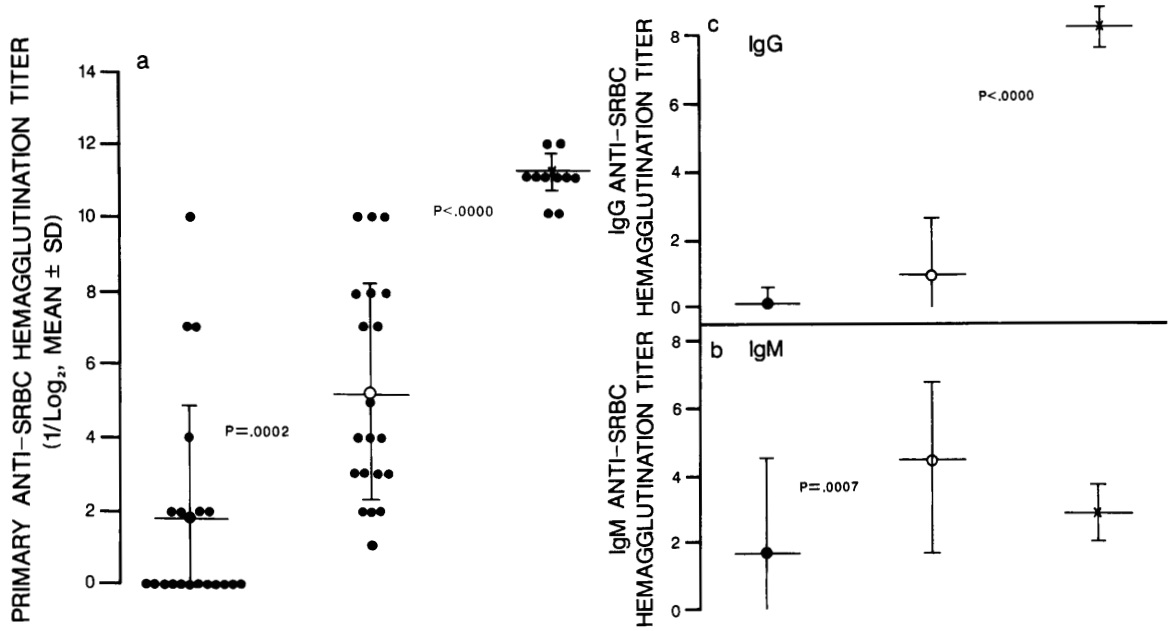

Figure 1. Primary antibody responses ($1/\log_2$) to SRBC by XID mice fed the control diet (●, n = 20, left columns) or the same diet supplemented with 0.4% *myo*-inositol (○, n = 20, middle columns), and immunocompetent mice fed the control diet (X, n = 10, right columns). (a) Total antibody (untreated serum). (b) IgM. (c) IgG. n = number of mice.

in the spleens of XID mice 5 days after a primary injection of SRBC; the XID mice had 10 times fewer splenic IgM PFC than did the immunocompetent controls (Fig. 2).

XID mice fed the diet supplemented with inositol had significantly higher titers of IgM antibody after rechallenge with SRBC than did XID mice or the immunocompetent controls given the standard diet (Fig. 3); no significant differences in IgG titers were noted among the groups.

Discussion

In summary, the studies on immunocompetent mice revealed that (i), *in vivo,* dietary *myo*-inositol (4 mg/g) ac-

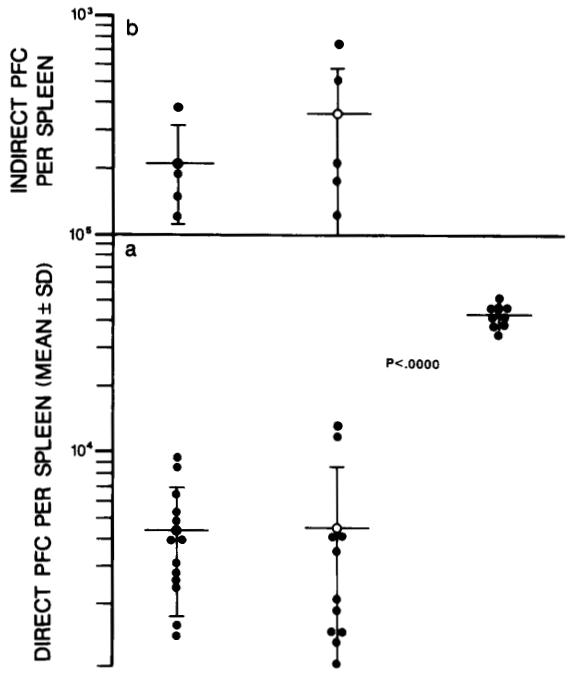

Figure 2. Anti-SRBC antibody-forming cells (PFC) in the spleens of XID mice fed the control diet (●, n = 13, left column) or the same diet supplemented with 0.4% *myo*-inositol (○, n = 11, middle column), and immunocompetent mice fed the control diet (X, n = 10, right column) 5 days after the primary injection of SRBC. (a) direct PFC (IgM). (b) Indirect PFC (IgG).

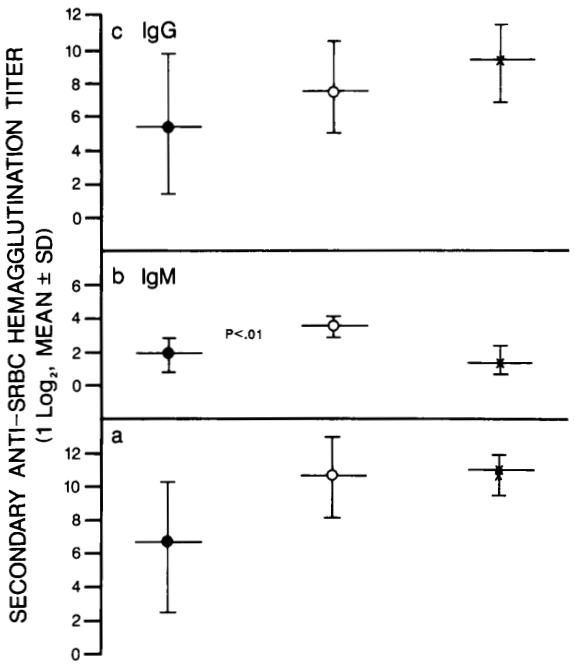

Figure 3. Secondary antibody responses ($1/\log_2$) to SRBC by XID mice fed the control diet (●, n = 5, left column) or the same diet supplemented with 0.4% *myo*-inositol (○, n = 5, middle column), and immunocompetent mice fed the control diet (X, n = 5, right column). (a) total antibody (untreated serum). (b) IgM. (c) IgG.

celerated the rate of appearance of IgM PFC and produced a modest increase in IgM hemagglutinating titers; (ii) in vitro, *myo*-inositol (4 mg/ml) accelerated the rate of appearance of colonies of IgM anti-SRBC PFC and produced an increase in the number of PFC/colony; and (iii) exposure to inositol both *in vivo* and *in vitro* resulted in a diminished number of PFC/focus following a primary *in vitro* response (IgG:IgM PFC ratio unchanged) and a more severe depression in PFC/colony (IgG > IgM PFC) following a secondary response. Thus, *myo*-inositol would appear to push naïve B cells toward terminal differentiation, accelerating and amplifying the IgM response but having little or no effect on the early conversion to IgG PFC. Exposure of B cells to *myo*-inositol during both primary and secondary responses to SRBC resulted in no change in the number of colonies of PFC detected at the end of the secondary response, but there was a marked decrease in the numbers of IgM and IgG PFC/colony, suggesting that replication during the secondary response was impaired and/or the life spans of the PFC were decreased.

In the studies on XID immunodeficient mice, the group fed a diet supplemented with 0.4% *myo*-inositol had modestly but significantly increased levels of IgM, but not IgG, anti-SRBC hemagglutinating antibody after primary and secondary immunizations. Too few mice were tested to be certain that the *myo*-inositol did not have a minor effect on IgG production during the secondary response.

However, despite having higher titers of agglutinating antibody, the XID mice fed the diet supplemented with *myo*-inositol did not have more PFC per spleen than the XID controls 5 days after initial challenge with SRBC. The studies in immunocompetent mice described above show that, *in vitro* and *in vivo, myo*-inositol accelerates the rate of PFC production early in the primary response to SRBC but does not affect the ultimate peak number of spleen PFC. Therefore, the results achieved in the XID mice are consistent with those noted in immunocompetent animals.

Mouse X-linked immunodeficiency is due to a mutation in Bruton's cytoplasmic tyrosine kinase that results in a failure of B cells to become phenotypically and functionally mature (11–13). B cells from XID mice do not respond to thymus-independent antigens (12), have reduced IgM and IgG_3 serum immunoglobulins (14), and have abnormal responses to several activation signals, such as immunoglobulin cross-linking (12), interleukin-5 (15), and interleukin-10 (16). This suggests that B cells of XID mice do not respond to essential signals for B cell proliferation, activation, and maturation.

The murine Btk gene has been mapped to the XID locus of the X chromosome, and sequencing of the gene has identified a missense mutation that might alter its role in signal transduction (6, 7). Cytoplasmic tyrosine kinases such as Btk are involved in lymphocyte signal transduction pathways by coupling with surface receptors. Important roles for Src and Syk/ZAP70 subfamily members have been shown in B and T cell receptor signal transduction (17). In addition, cytoplasmic tyrosine kinases may perform essential regulatory functions—for instance, CsK modulates the activity of Src-subfamily kinases and downregulates the signal transduction pathways in which they participate (18).

myo-Inositol 1,4,5-triphosphate (IP_3) is a ubiquitous second messenger that couples agonist stimulation of a wide variety of cell surface receptors to the mobilization of intracellular calcium (19, 20). IP_3 is released from phosphatidylinositol in response to activation of certain receptor-associated or cytoplasmic tyrosine kinases. Although the mechanism(s) of Btk signal transduction is still incompletely understood, it is not unreasonable to assume that the generation of the second messenger IP_3 may play some role. If this assumption is correct, the effects of dietary inositol observed in these experiments could be explained by positive or negative (21) effects of this agent or a metabolite on the inositol second messenger system. From this it would follow that "second messenger" therapy, stimulatory or inhibitory, may be feasible in certain disorders.

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