

THE EFFECT OF 1,2-DIHYDRO-6-ETHOXY-2,2,4-TRIMETHYLQUINOLINE (SANTOQUIN) ON THE *IN VITRO* PRIMARY IMMUNE RESPONSE OF NZB/NZW MICE

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ABSTRACT

The antioxidant Santoquin has been shown to prolong the average life span of various organisms, to prevent tumor development in some model systems, to delay the onset of autoimmune disease in mice, and to enhance the immune response in C₃HeB/FeJ mice. The intention of this study is to determine the effect of Santoquin on the primary immune response of New Zealand Black×New Zealand White (NZB/NZW) mice.

NZB/NZW mice were fed a diet supplemented with 0.25 percent weight Santoquin for a period of 10 weeks. At the end of the feeding period, the mice were sacrificed and the spleen cells cultured four days with sheep red blood cell antigen by a modification of the Mishell-Dutton system. Antibody production was assayed by the Jerne hemolytic plaque technique. Mice fed the supplemented diet showed a significant augmentation of the primary immune response when compared to the control group. This augmentation suggests that Santoquin may protect the components of the immune system from the deleterious effects of free radical reactions.

INTRODUCTION

The New Zealand Black×New Zealand White (NZB/NZW) hybrids were chosen for this study primarily because they tend to lose their ability to respond to foreign antigens much earlier in life than do mice from other stocks (Ohsugi et al. 1978). Relatively few studies have dealt with the effect of antioxidants on the immune system. Harman (1980) showed that the manifestations of autoimmune disease could be slowed and reduced by the addition of 0.25%w vitamin E, 0.25%w Santoquin, and 1.0%w sodium hypophosphite to the diet of NZB mice. Ohsugi et al. (1978) showed that the water soluble antioxidant N-(2-Carboxyphenyl)-4-Chloroanthranilic acid disodium salt prevented autoimmune kidney disease in NZB/NZW F1 hybrid mice. Harman (1977) did an extensive study on the effect of various antioxidants on the

immune response. He found that 0.25%w vitamin E and 0.25%w Santoquin augmented both the humoral and cell mediated response of C₃HeB/FeJ mice. It was shown that from the age of 28 to 88 weeks, vitamin E and Santoquin enhanced the humoral and cell mediated responses by as much as 266% of the control groups. It was also shown that the *in vivo* humoral response could be enhanced by BHT, Santoquin, 2-MEA, and sodium hypophosphite. However, other antioxidants such as BHA, probucol, and sodium bisulfite failed to increase the humoral response. It was also demonstrated that Santoquin and sodium hypophosphite would not act synergistically to enhance the response. Thus, some antioxidants may enhance the immune response of C₃HeB/FeJ mice and others may not. The same may be said for the ability of antioxidants to inhibit autoimmune manifestations.

The intention of this study is to determine the effect of Santoquin on the *in vitro* primary immune response of NZB/NZW mice.

MATERIALS AND METHODS

Age matched NZB/NZW male mice were used in these experiments. Mice were watered ad libitum and fed 3-5 grams per mouse per day of a mash feed for 10 weeks prior to initiation of the experiment. A mash feed obtained from Wayne Research Animal Diets was used to facilitate mixing of the Santoquin antioxidants supplement. Each week a premix containing two percent by weight (2%w) of the antioxidant supplement was prepared and subsequently diluted to the 0.25%w concentration used to feed the experimental mice. Nonsupplemented feed was obtained from the same batch to feed the control mice.

A review of the literature indicated that there was no standing data on which the level of Santoquin would be most effective in augmenting the immune response (Harman 1981, 1980); therefore, an initial experiment was set up using nine NZB/NZW mice in which the mice were randomly assigned to three groups. Three mice made up a control group, three mice comprised a group fed 0.5%w

Santoquin in their diet, and three mice were fed the diet supplemented with 0.25%w Santoquin. The mice were started at seven weeks of age on their respective diets and were fed for 10 weeks to ensure both a proper exposure to Santoquin and immunological maturity. The 0.5%w group showed a three-fold augmentation, and the 0.25%w group showed a 5.6-fold augmentation of the primary immune response. For both conservation of Santoquin and immune augmentation reasons, the 0.25%w level of Santoquin was chosen for the primary experimentation. Thirty-six 8-week old NZB/NZW mice were randomly assigned to a control group and an experimental group. The control group was fed a mash diet with no Santoquin supplementation, and the experimental group was fed a diet supplemented with 0.25%w Santoquin. The mice were fed their respective diets until time of sacrifice. At 18, 19, and 20 weeks of age, mice were sacrificed, and spleen cells were pooled from each group and cultured four days. At the end of the culture period, cell suspensions were prepared following the technique described by Mishell and Dutton (1967).

The hemolytic plaque assay described by Jerne et al. (1963) was used to determine the number of antibody producing cells. The data from the initial study using 17-week-old NZB/NZW mice and 0.5%w and 0.25%w Santoquin supplemented diets was subjected to a Model I completely randomized analysis of variance. The Student-Neumann-Kuels multiple range test was used to determine significant differences among the treatment groups (Scheffler 1980). The primary data from NZB/NZW mice, 18, 19, and 20 weeks old, fed a diet supplemented with 0.25%w Santoquin was subjected to a 2x3 factorial analysis of variance.

RESULTS AND DISCUSSION

All plaque-forming cell data is expressed as the number of antibody-producing cells per 1.5×10^7 viable cells and represents the *in vitro* primary humoral immune response. All plaque-forming cell data is corrected for background plaques.

Table 1 and Figure 1 show the results of the initial study performed using NZB/NZW mice to determine the optimal level of Santoquin for augmentation of the immune response.

The control group mean anti-SRBC PFC was 109.33. The 0.5%w Santoquin supplemented diet group mean anti-SRBC PFC was 328.00, and the 0.25%w group mean was 615.00. The data from this study was subjected to analysis of variance and the Student-Neumann-Kuels multiple range test (Scheffler 1980). Both experimental groups showed a significantly ($p < 0.01$) enhanced response over that of the controls (Table 2). Also, the experimental groups were significantly ($p < 0.01$) different from one another. From this data, the 0.25%w level of Santoquin

Table 1. Initial studies of the effect of 0.5%w and 0.25%w Santoquin supplemented diets on the *in vitro* primary immune response of 17-week-old NZB/NZW F1 hybrid mice.

Santoquin	PFC/ 1.5×10^7 cells
0 (control)	109.33 3.76 ^a
0.5%w	328.00 11.27*
0.25%w	615.00 52.05*

^aValues represent the x corrected for the background plaques the SE of the anti-SRBC PFC response with an n of 3.

*Statistically different from control ($p < 0.01$) and statistically different from one another ($p < 0.01$).

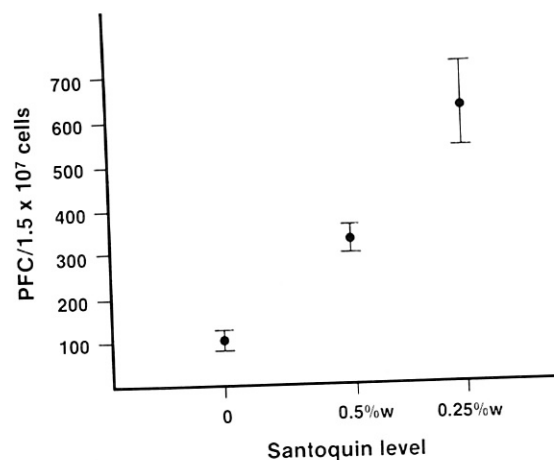


Figure 1. The effect of 0.5%w and 0.25%w Santoquin supplemented diet on the *in vitro* primary immune response of 17-week-old NZB/NZW F1 hybrid mice. Each bar represents the anti-SRBC PFC response from three cultures. Each mean represents the anti-SRBC response of four cultures.

was chosen for continuation of the study.

The hemolytic plaque assay results of the primary study are shown in Table 3 and Figure 2. The mean number of PFC at 18 weeks of age was 107.67 for the control group and 579.17 for the experimental group. At 19 weeks, the mean of PFC was 109.50 for the controls and 582.17 for the experimental group. Again, the augmentation is well over 5 times. At 20 weeks of age, the mean of PFC was 103.50 for the control group and 525.17 for the experimental group. At this age, the enhancement

Table 2. ANOVA table of the initial studies of the effect of 0.5%w and 0.25%w Santoquin supplemented diets on the primary immune response of 17-week-old NZB/NZW F1 hybrid mice.

Source of variation	df	SS	MS	F-ratio
Treatment (diet)	2	385,883	17,941.5	58.302*
Error	6	17,798	2,966.3	

*Statistically significant ($p < 0.01$; $F = 10.92$ at 2,6 df).

df=degrees of freedom

SS=Sum of Squares

MS=Mean Squares

was slightly over five times than that of the control group. Thus, at each age, mice fed a diet supplemented with 0.25%w Santoquin showed an enhancement of the primary immune response when compared to mice fed a diet not supplemented with Santoquin.

The hemolytic plaque assay data from the above study was subjected to a 2x3 factorial analysis of variance to determine the statistical significance for the effects of age, supplemented diet, and the interaction of age and diet on the immune response. Age and interaction of age and diet were not significant factors in this study. However, mice fed a diet supplemented with 0.25%w Santoquin showed a significant ($p < 0.01$) augmentation of the primary immune response when compared to mice fed an unsupplemented diet (Table 4).

The addition of Santoquin as a dietary antioxidant supplement augmented the primary humoral response of male NZB/NZW mice. In each case the experimental groups showed augmentations ranging from three times to well over five times. Harman (1977) reported an almost three-fold enhancement of the primary response in female C3HeB/FeJ mice when fed a diet supplemented with Santoquin. The 0.25%w Santoquin diet supplement enhanced the response much better than the 0.5%w supplement in this study. However, both levels significantly augmented the humoral response when compared to a nonsupplemented diet. The results of this study indicate that Santoquin at both 0.5%w and 0.25%w has the ability to enhance the *in vitro* primary immune response.

The enhancement exhibited by Santoquin is possibly due to the protection of various elements of the immune system afforded by Santoquin. Because Santoquin is a lipid-soluble antioxidant, the protection provided is probably associated with the lipids of the immune system. Nevertheless, it cannot be ruled out that other systems are protected by Santoquin's antioxidant quality. It is also possible that Santoquin may act to prime the immune

Table 3. Primary study of the effect of Santoquin supplemented diet on the *in vitro* primary immune response of F1 hybrid NZB/NZW mice.

Age	Control		Experimental		
	Weight	PFC	Weight	PFC	%C
18	39.43 0.59 ^a	107.67 4.66 ^b	37.97 1.12	579.17 26.92*	550 ^c
19	40.78 1.09	109.50 5.04	39.76 0.94	582.17 11.06*	532
20	43.76 1.78	103.50 4.73	44.53 0.74	525.17 37.25*	507

^aValues represent the $\bar{x} \pm SE$ of the weight in grams with an n of 6.

^bValues represent the \bar{x} corrected for background plaques $\pm SE$ of the anti-SRBC PFC response with an n of 6.

^c%C=#PFC experimental/#PFC controlx100.

*Statistically different from the control ($p < 0.01$).

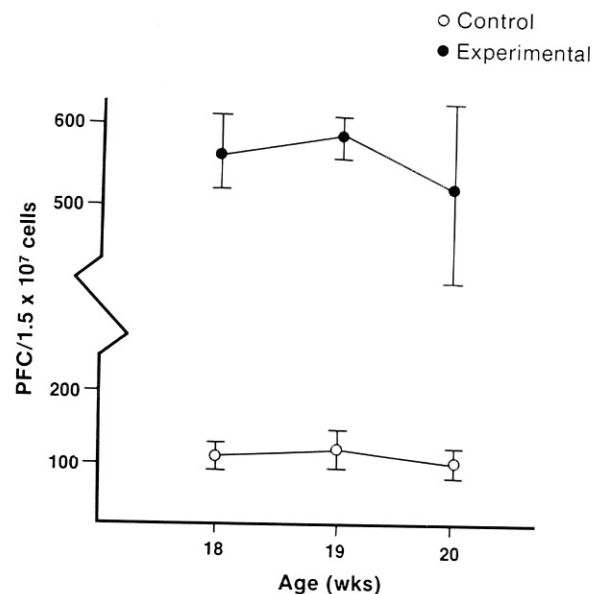


Figure 2. The effect of Santoquin on the primary immune response. Bars represent one standard error. Each mean represents the anti-SRBC response of six cultures.

system for a response to SRBC; however, there are no studies which show any cross-reactivity between Santoquin and SRBC.

The most fundamental lipid element of the immune system is the cell membranes of the lymphocytes and accessory cells. Free radical reactions could potentially damage lipids of the cell membrane and thus alter the sites

Table 4. ANOVA table of the primary studies for the effect of 0.25%w Santoquin supplemented diet and age on the primary immune response.

Source of Variation	df	SS	MS	F-ratio
Columns (diet)	1	1,865,500.70	1,865,500.70	809.60*
Rows (age)	2	7,375.73	3,687.86	1.60
Interaction (diet x age)	2	5,085.72	2,542.86	1.10
Error	30	69,126.83	2,304.22	

*Statistically significant ($p < 0.01$; $F = 7.56$ at 1,30 df).

df=degrees of freedom

SS=Sum of Squares

MS=Mean Squares

in which hydrophobic molecular parts are anchored. Santoquin may act to preserve the cell surface characteristics so important in cellular communication in the humoral response. Both macrophages and lymphocytes rely heavily on the integrity of the cell membrane for their proper function. Macrophages use the cell membrane not only to engulf antigens but also to present the antigens to lymphocytes and to secrete signal molecules known as monokines. It is a very active cell and is an essential link in the proper immune response. Santoquin may augment the response by protecting the lipid components of the macrophage.

T and B lymphocytes are also very active during the humoral response and may be subject to the degradative action of free radicals on cellular lipids. The Ly-123, Ly-1, and Ly-23 cells must be maintained in a delicate balance to respond to foreign material. Santoquin may augment the primary response by protecting the lipid components of the T-lymphocytes. The B-cell not only must differentiate into an antibody-producing plasma cell but also must be able to respond to signals from the inducer and repressor cells. The plasma cell is a large cell possessing an extensive endoplasmic reticulum which is responsible for antibody productions. Jerne et al. (1963) estimated that the plasma cell produces 5,000 antibody molecules per second. If the nuclear envelope or endoplasmic reticulum of such an active cell is damaged by free radical reactions, the effects could be devastating. However, the ability to augment the immune response

probably lies in the ability of Santoquin to protect lipid elements of B and T lymphocytes and macrophages.

Other components of the immune system may also be afforded protection by Santoquin. The stem cells and progenitor cells in the bone marrow are responsive to inducing factors and hormone-like substances such as thymopoietin. Santoquin may protect the stem and progenitor cells and/or the cells of the hemopoietic microenvironments which produce the hormones.

Based on the results of this study, it was found that Santoquin enhanced the primary humoral response by more than five times than that of the control group. The means by which Santoquin augments the response could not be identified by this study. However, the antioxidant quality of Santoquin probably protects the various components of the immune system, including the plasma membrane and lipid-bound organelles of the cells, the tissue of the lymphoid organs, and perhaps other cellular components.

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