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EFFECT OF INDUCTION OF T-CELL-DEPENDENT ANTIBODY WITH SHEEP RED BLOOD CELLS ON P-450-DEPENDENT AND -INDEPENDENT XENOBIOTIC METABOLIZING ENZYMES*

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Abstract—The effect of an antigenic challenge with sheep red blood cells (SRBC) on the activities of cytochrome P-450-dependent and -independent xenobiotic metabolizing enzymes and on lipid peroxidation in the liver was investigated. The studies were carried out using three mouse strains of C57Bl/10 and three strains of C3H backgrounds which are cogenic, differing genetically at the H-2 complex. The basal levels of aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin *O*-deethylase (7-Ec) were different among congenic strains. The activity of 7-Ec was lower in C3H background mice than in B10 background mice. Similarly, the difference due to the strain and the H-2 locus was detected in the activities of P-450-independent enzymes such as malathion and diethyl succinate carboxylesterases, glutathione *S*-transferase, and epoxide hydrolases in microsomal and cytosolic fractions. The degree of immune responsiveness in these mice was determined by a plaque forming cell assay. Within the same background, the H-2^b mouse strain was a high responder and the H-2^k a low responder to SRBC. However, treatment with SRBC had no significant depressive effect on P-450-dependent enzyme activities except in C3H/He. Activity of AHH was suppressed in C3H/He mice. Treatment with SRBC had no effect on P-450-independent enzyme activities except for malathion carboxylesterase: the activity was increased in C3H/He and C3H.JK, whereas it was decreased in B10. The basal level of lipid peroxidation was lower in C3H/He and C3H.JK. The treatment produced a significant enhancement in lipid peroxidation in C3H/He, B10 and B10.BR ($P < 0.05$) with a concomitant increase in xanthine oxidase activity ($P < 0.05$). Thus, the present study revealed that a specific antigenic challenge, unlike non-specific immunostimulants (e.g. poly IC, endotoxin), does not necessarily inhibit P-450-dependent xenobiotic metabolizing enzymes even though antigen challenge increased XO activity and lipid peroxidation. The possible roles of an increase in lipid peroxidation and xanthine oxidase activity in immune response to SRBC and xenobiotic metabolizing enzymes are discussed.

Accumulated evidence indicates that a reciprocal relationship exists between the inhibition of P-450-dependent mixed-function oxidases (MFOs) and the immunopotentiation following the treatment of animals with immunostimulants. Several broad-spectrum non-specific immunostimulants reduce the activities of P-450-dependent MFOs [1], while many MFO inducers such as 3-methylcholanthrene [2], phenobarbital [3], and β-naphthoflavone [4] are known to be immunosuppressive. More detailed studies of the effect of non-specific immunostimulants on MFOs and related enzymes have revealed that the immunostimulants, such as polyriboinosinic polyribocytidylic acid (poly IC), an interferon inducer, and an interferon *per se*, inhibit MFOs and induce xanthine oxidase (XO) simultaneously [5, 6]. Furthermore, increased lipid peroxidation occurred together with these changes [7, 8].

There is some evidence that free radicals and/or lipid peroxidation are causally related to the inhibition of P-450-dependent MFOs produced by immunostimulants [7–9]. The present study was conducted in order to determine whether a specific antigenic immunostimulation could cause inhibitions of P-450-dependent MFOs, increase in XO, and/or enhancement of lipid peroxidation similar to those seen with broad-spectrum immunostimulants. In the present study, we selected sheep red blood cells (SRBC) as a specific antigen because SRBC has been known to induce T-dependent antibody and to elicit an immune response regulated by H-2 complex, the major histocompatibility complex in mice [10]. Three strains of C57Bl/10 mice and three strains of C3H mice which are congenic at their H-2 locus were used for the study. These animal models would allow us to examine whether, and to what extent, H-2 locus influences stimulation of immune responses, inhibition of MFOs, induction of XO, and/or enhancement of lipid peroxidation.

MATERIALS AND METHODS

Animals. Young congenic male mice (3 months old) of C57Bl/10 background, B10 (H-2^b), B10.BR (H-2^k) and B10.RIII (H-2^r), and C3H background, C3H/He (H-2^k), C3H.JK (H-2^j), and C3H.SW (H-2^b), were used. All the mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Animals were housed in plastic cages on wood

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shavings in a room designed to control temperature at 23° relative humidity at about 50%, and a 12-hr photocycle. Animals were fed with commercially available laboratory chow (Wayne Rodent Blox, Continental Grain Co., Chicago, IL) *ad lib.* Mice were immunized with SRBC by administration of 1% SRBC in Hanks' balanced salt solution (1 ml, i.p.) around 9:00 a.m. Control animals received 0.9% NaCl solution. On day 5 following immunization, mice were killed by cervical dislocation between 8:30 a.m. and 9:30 a.m., and the liver and spleen were collected. The rationale for the protocol of the immunization is that the method results in the maximal immune response as measured in the plaque forming cell (PFC) assay [11].

PFC assay. Single cell suspensions were prepared from the spleens in 10 ml of Hanks' balanced buffer. After two washes, cells were resuspended at a concentration of 20×10^6 cells per ml. Fifty microlitres of this cell suspension was mixed with 25 μl of a 10% suspension of SRBC and 25 μl of diluted guinea pig serum. Plaques were measured after a 1-hr incubation at 37° in paraffin-sealed chambers [12].

Enzyme preparation and enzyme assays. Livers were removed immediately after sacrifice, washed with ice-chilled isotonic saline solution, and homogenized in ice-cold KCl–Tris buffer solution (1.15% KCl in 0.02 M Tris–HCl at pH 7.4) with a Potter–Elvehjem homogenizer. The postmitochondrial supernatant fraction, prepared by centrifuging the homogenate at 9000 *g* for 20 min, was further centrifuged at 105,000 *g* for 60 min to collect the microsomal pellet and the cytosolic fraction. The microsomal pellet was washed once with the homogenizing buffer and resuspended in KCl–Tris buffer.

The activities of microsomal aryl hydrocarbon hydroxylase (AHH) and 7-ethoxycoumarin *O*-deethylase (7-Ec) were assayed fluorometrically by following the rate of formation of hydroxy metabolites benzo[*a*]pyrene [13] and umbelliferone [4] respectively. *p*-Nitroanisole *O*-demethylase (*p*-NA), malathion and diethyl succinate carboxylesterases were assayed spectrophotometrically [14, 15]. P-450 content and cytochrome *c* reductase activity in microsomes were determined by the methods of Omura and Sato [16] and Yasukochi and Masters [17] respectively. Epoxide hydrolase activities in microsomal and cytosolic fractions were determined by following the conversion of tritiated styrene oxide (2 μM, sp. act. 84 mCi/mmole, Amersham International, U.K.) to styrene glycol according to the procedure of Oesch *et al.* [18]. The activity of glutathione *S*-transferase was determined spectrophotometrically with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate [19]. Lipid peroxidation in the liver homogenate was measured *in vitro* by following the formation of malondialdehyde (MDA) by the thiobarbituric acid method using an extinction coefficient of $156\,cm^{-1}\,mM^{-1}$ [20]. XO exists as two interconvertible forms [21], an oxidase and an NADH-dependent dehydrogenase. The activities of these two forms were assayed after overnight dialysis of the cytosolic fraction at 4° against 1.15% KCl. The activities were determined by the method of Della Corte and Stirpe [21]. The total and specific activities of glucose-6-phosphatase (G-6-P) in homogenate and microsomes were determined by the method of Traiger and Plaa [22]. Protein concentration was determined by the method of Sedmak and Grossberg [23].

Statistical analysis. All data are presented as mean ± S.E. Student's *t*-test was conducted to detect differences of mean activities of AHH, 7-Ec, *p*-NA and XO and mean of lipid peroxidation in the treated animals in comparison with counterpart controls. The activities of cytochrome P-450 content, cytochrome *c* reductase, epoxide hydrolase, glutathione *S*-transferase, and carboxylesterases in control and treated animals of different strains were compared by using an analysis of variance followed by Duncan's multiple range test [24]. Comparisons of basal level activities of AHH, 7-Ec, p-NA, XO and lipid peroxidation among different strains were carried out by using an analysis of variance followed by Duncan's multiple range test [24]. A $P < 0.05$ was considered significant.

RESULTS

SRBC specific PFC response. As shown in Table 1, there were differences in the PFC responses between

Table 1. Summary of significant effects* of SRBC on PFC response *in vivo*, P-450-dependent MFO activities, lipid peroxidation and xanthine oxidase activity

Strain	Haplotype	SRBC-specific PFC†	AHH‡	7-Ec‡	*p*-NA‡	Lipid peroxidation‡	XO‡
B10	H-2^b	857 ± 25§	NS	NS	NS	1.35	1.42
B10.BR	H-2^k	507 ± 27‖	NS	NS	NS	1.72	1.50
B10.RIII	H-2^r	702 ± 25¶	NS	NS	NS	NS	NS
C3H/He	H-2^k	391 ± 9‖	0.77	NS	NS	2.35	2.85
C3H.JK	H-2^j	245 ± 29**	1.59	1.38	1.48	NS	0.78
C3H.SW	H-2^b	468 ± 4‖	NS	NS	NS	NS	NS

* $P < 0.05$.

† Plaque formation is expressed per 2×10^6 spleen cells. Each set is the mean ± S.E. of five animals with triplicate measurements for each animal.

‡ NS: Not significant in comparison with controls. A number indicates a ratio of the mean value in animals treated with SRBC over that in control animals.

§–** Statistical comparisons between groups were done by one-way analysis of variance followed by Duncan's multiple range test. Treatment means in vertical columns not followed by the same superscript are significantly different from each other ($P < 0.01$).

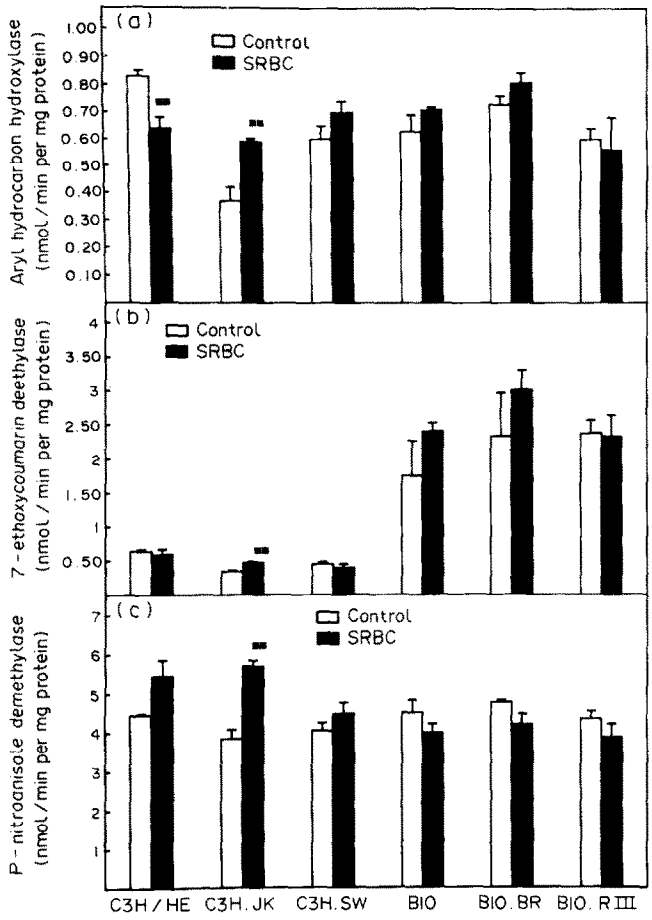

Fig. 1. Effect of treatment with SRBC on the activities of (a) aryl hydrocarbon hydroxylase, (b) 7-ethoxycoumarin deethylase and (c) *p*-nitroanisole demethylase. Bars depict mean ± S.E. of five animals per strain. Key: (**) significant difference from corresponding control value ($P < 0.01$), analyzed by Student's *t*-test.

B10 and C3H mice following treatment with SRBC. The order of the magnitude of the response was B10 (H-2^b) > B10.RIII (H-2^r) > B10.BR (H-2^k) > C3H.SW (H-2^b) > C3H/He (H-2^k) > C3H.JK (H-2^j). The response appeared to be greater in mice with B10 background than C3H. Among the strains with the same background, H-2 haplotypes appeared to be a primary factor in determining the magnitude of responsiveness; the H-2^b was a high responder whereas the H-2^k was a low responder in both B10 and C3H mice.

Effect of immunization with SRBC on P-450-dependent MFOs. As shown in Fig. 1, the effects of SRBC on AHH, 7-Ec and *p*-NA diversely varied depending on the strain and the H-2 haplotypes. The activity of AHH was decreased in C3H/He but was increased in C3H.JK following the immunization. 7-Ec and p-NA activities were increased in C3H.JK but were not changed in any other strains following immunization with SRBC (Fig. 1). The effects of SRBC treatment on MFOs are summarized in Table 1. There were some differences in basal level activities of MFOs: the activity of AHH was significantly lower in C3H.JK in comparison with other strains, and the activity of 7-Ec of C3H background mice was lower than in B10 background mice. There were no changes in P-450 content and cytochrome *c* reductase activity after SRBC treatment (Table 2). It was evident that B10 background mice uniformly had higher P-450 contents than C3H background mice (Table 2). The recoveries of microsomal fraction, as indicated by the ratio of the total activity of G-6-P in the microsomal over that in the cytosolic fractions, were essentially the same in the control and the treated animals of each strain (data not shown).

Effects of immunization with SRBC on lipid peroxidation and xanthine oxidase activity. As shown in Fig. 2, lipid peroxidation measured by the production of MDA was enhanced in the treated animals of C3H/He, B10 and B10.BR strains. The increase in activity of total XO due to the treatment with SRBC was also observed in the treated animals of C3H/He, B10 and B10.BR strains (Fig. 2). The effects of treatments with SRBC are summarized in Table 1. There was a striking difference in the basal levels of lipid peroxidation between groups of mice: in C3H/He and C3H.JK, basal levels of lipid peroxidation were lower than in other strains ($P < 0.05$; Fig. 2). The basal levels of XO activity were lower in C3H/

Table 2. Effects of immunostimulation with SRBC on P-450 content cytochrome *c* reductase, and P-450-independent xenobiotic metabolizing enzymes

Strain	H-2 haplotype	Treatment	P-450 (nmoles/mg protein)	Cytochrome *c* reductase (nmoles/min/mg protein)	Epoxide hydrolase (nmoles/min/mg protein) Cytosol	Microsome	Glutathione-*S*-transferase (nmoles/min/mg protein)	Carboxylesterase (nmoles/min/mg) Malathion	Diethyl succinate
B10	$H\text{-}2^b$	Control	1.09 ± 0.104*	358 ± 25.5†‡§‖	3.95 ± 0.189*†	1.86 ± 0.071†‡	6.50 ± 0.195*	30.8 ± 2.87*	1320 ± 159‡
		SRBC	1.15 ± 0.033*	386 ± 47.7†‡§	3.91 ± 0.368*†	1.37 ± 0.286‡	5.46 ± 0.319*	13.8 ± 0.963§	1247 ± 69.5‡
B10.BR	$H\text{-}2^k$	Control	1.19 ± 0.057*	407 ± 20.9*†‡	2.64 ± 0.291†	2.34 ± 0.532†‡	4.27 ± 0.291*	14.6 ± 1.04§	1348 ± 86.0‡
		SRBC	1.05 ± 0.045*	375 ± 29.0†‡§	3.22 ± 0.171*†	2.35 ± 0.561†‡	4.08 ± 0.202*	12.2 ± 0.782§	1259 ± 68.0‡
B10.RIII	$H\text{-}2^r$	Control	1.09 ± 0.039*	269 ± 7.00§‖	2.77 ± 0.488*†	3.61 ± 0.847*†	6.54 ± 0.602*	12.5 ± 0.917§	1242 ± 80.0‡
		SRBC	1.17 ± 0.129*	244 ± 3.13‖	3.29 ± 0.613*†	1.72 ± 1.32†‡	4.52 ± 0.428*	14.7 ± 0.333§	1243 ± 84.0‡
C3H/He	$H\text{-}2^k$	Control	0.708 ± 0.053†	424 ± 30*†	2.87 ± 0.434*†	1.66 ± 0.394†‡	3.88 ± 0.528*	21.9 ± 1.27‡	1962 ± 119*†
		SRBC	0.660 ± 0.035†	516 ± 47.6*	3.36 ± 0.385*†	2.07 ± 0.414†‡	4.39 ± 0.550*	29.1 ± 1.29*†	2139 ± 255*
C3H.JK	$H\text{-}2^j$	Control	0.791 ± 0.033†	274 ± 26.8§‖	3.72 ± 0.606*†	1.79 ± 0.148†‡	6.82 ± 0.249*	14.8 ± 1.16§	1327 ± 55.6‡
		SRBC	0.722 ± 0.023†	289 ± 20.8§‖	4.62 ± 0.650*	3.24 ± 0.324*†‡	4.93 ± 0.689*	24.6 ± 1.55†‡	1582 ± 142†‡
C3H.SW	$H\text{-}2^b$	Control	0.619 ± 0.031†	302 ± 22.0†§‖	4.27 ± 0.434*†	3.27 ± 0.287*†‡	5.93 ± 1.95*	24.4 ± 0.941‡	1633 ± 107*†‡
		SRBC	0.715 ± 0.032†	343 ± 12.0†‡§‖	3.46 ± 0.480*†	4.35 ± 0.480*†	6.05 ± 1.36*	24.1 ± 0.99†‡	1375 ± 197‡

Values represent the mean ± S.E. (N = 5).
*-‖ Values in a vertical row with the same superscript are not significantly different at $P > 0.01$.

He, B10 and B10.BR than in other mice ($P < 0.05$; Fig. 2). There was a significant positive correlation between magnitude of increases of lipid peroxidation and magnitude of increases of XO activity after treatments with SRBC in pooled animals of C3H/He, B10 and B10.BR (data not shown; $r = 0.70$; $P < 0.01$; $N = 15$). Although SRBC increased XO activity and enhanced lipid peroxidation, a qualitative relationship was not observed between the degree of immune responses and the magnitude of lipid peroxidation or increased XO activity (data not shown).

Effect of immunization on P-450-independent enzymes. As summarized in Table 2, treatment of mice with SRBC had no effect on the activity of diethyl succinate carboxylesterase. Similarly, the activity of glutathione *S*-transferase toward CDNB or epoxide hydrolase in microsomes or cytosol was not affected by the treatment. In contrast, malathion carboxylesterase was changed by treatment with SRBC: the activity was decreased in B10 and increased in C3H/He and C3H.JK.

DISCUSSION

A large number of non-specific immunostimulants are known to alter the normal rate of xenobiotic metabolism by depressing the MFOs. These are *Bordetella pertussis* vaccine [25], BCG [26], dextran [27], trypan blue [28], tilorone [29], poly IC [29, 30] and host-mediated antitumor drugs such as OK 482 [31]. When any of the above stimulants is introduced to animals, a number of cellular interactions take place and thereby trigger a vigorous polyclonal stimulation of the immune system: *Bordetella pertussis* activates macrophage and several other related mechanisms; dextran and trypan blue activate the reticuloendothelial system; and poly IC and tilorone induce interferon. In the present study we used SRBC as a T-cell-dependent antigen to induce antibody formation through T cell and B cell cooperation. Such a response is largely regulated by the H-2 locus [10]. The H-2 locus seems to be a primary factor for determining PFC responses among strains of the same background. Thus, it was possible to examine the influence of H-2 locus on immunostimulation and alteration of MFOs due to antibody formation. Although the degree of immune response measured by the PFC assay varied diversely depending on H-2 haplotype (Table 1), no suppressive change was detected in the activities of MFOs following the challenge with SRBC except in C3H/He (Fig. 1, Table 1). On the contrary, MFOs were increased by the treatment with SRBC in C3H.JK. Therefore, antibody induction evoked by SRBC appeared not to necessarily depress MFOs. We believe this is the first paper which shows that an antibody response to a specific antigen challenge, unlike non-specific immunopotentiators, does not necessarily suppress the activities of MFOs. It has been suggested that the Ah locus, which regulates the inducibility of AHH and a part of which is assigned to chromosome 17 [32], has a close relationship with the H-2 locus which resides on chromosome 17 [33]. Furthermore, the regulatory loci of these genes have been speculated to overlap to some extent. This speculation was

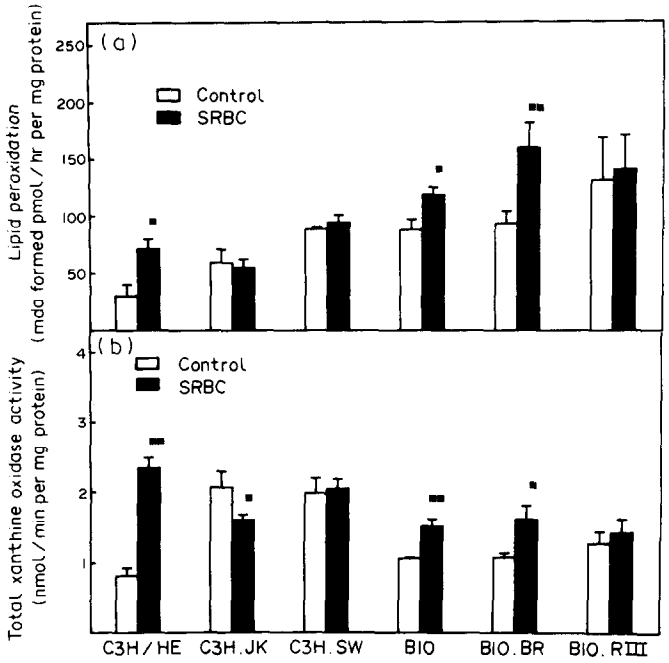

Fig. 2. Effect of treatment with SRBC on (A) lipid peroxidation and (B) xanthine oxidase activity. Bars indicate mean ± S.E. of five animals per strain. Key: (*) and (**) indicate significant differences from corresponding control values ($P < 0.05$ and $P < 0.01$ respectively), analyzed by Student's t-test.

derived from a clear-cut "dichotomy" which claims that most immunostimulators depress AHH and most of the AHH inducers act as immunosuppressants [34]. With regard to the basal level of AHH, the role of Ah locus is unknown. However, our present study suggests that the part of H-2 locus which regulates the specific immunostimulatory response (i.e. antibody production) has a suppressive effect on the activities of AHH in only a single strain of mice (C3H/He). In addition, the counterpart haplotype mice on a B10 background, B10.BR ($H\text{-}2^k$), did not show any suppression of AHH due to the SRBC treatment. Therefore, the suppression of MFOs due to specific immune responses such as SRBC cannot be explained by either background strain difference or H-2 haplotype.

The increase of XO activity following poly IC, interferon or virus treatment has been reported [5, 6]. Recently, an increase in XO activity has been causally related to free radical formation [9] or related to enhanced lipid peroxidation [7, 8]. In the present study, this idea is confirmed by the finding that there was a significant enhancement of lipid peroxidation and a concomitant increase of XO activity following the treatment of SRBC whenever either of them was increased. This finding indicates that there may be a cause–effect relationship between the two events. Although no quantitative correlation exists between degree of immune response and these events, these data support the view that the enhancement of lipid peroxidation and the increase of XO activity may be triggered by immune responses. In this regard, it is noteworthy that some antioxidants suppress the immune response by scavenging the hydroperoxides [35] that are capable of increasing lipid peroxidation.

Depression of MFOs may be caused by a limited class of immunostimulants such as poly IC, endotoxin and Triton. It has been proposed that there may be at least four mechanisms for decreasing the activity of MFOs due to non-specific immunostimulants: (1) depression of δ-aminolevulinic acid synthase together with an induction of hemeoxygenase [36, 37]; (2) acceleration of heme dissociation from P-450 [36]; (3) decrease in synthesis of apoprotein of P-450 [38]; and (4) free radicals and/or enhancement of lipid peroxidation [7–9]. Although enhancement of lipid peroxidation following the treatment with SRBC was observed in three strains, a significant suppression of P-450 was observed only in C3H/He. This might suggest that the magnitudes of the enhancement of lipid peroxidation and the increase in XO activity are important. As a matter of fact, the magnitude of lipid peroxidation and the increase in XO activity were less extensive in B10 and B10.BR than in C3H/He. If so, both increased activity of XO and lipid peroxidation, which are also found in animals treated with non-specific immunostimulants such as poly IC or inteferon [5, 6] may represent a more substantial change following immunostimulation than the apparent suppression of P-450-dependent MFOs. However, obviously this hypothesis needs further confirmation as to whether there is an involvement of mechanisms from (1) to (3) listed above after the immunization with SRBC. In addition, the apparent strain response differences to SRBC in XO activity and lipid peroxidation also need to be explained.

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