Biochemical Pharmacology, Vol. 37, No. 14, pp. 2765–2771, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

ROLE OF THE TRANSFER OF METABOLITES FROM HEPATOCYTES TO SPLENOCYTES IN THE SUPPRESSION OF *IN VITRO* ANTIBODY RESPONSE BY DIMETHYLNITROSAMINE

DONG-HYUN KIM,* KYU-HWAN YANG,* KIRK W. JOHNSON and MICHAEL P. HOLSAPPLE†

* Korea Advanced Institute of Science and Technology, Seoul, Korea, and Department of Pharmacology and Toxicology, Medical College of Virginia (V.C.U.), Richmond, VA 23298, U.S.A.

(*Received* 24 *August* 1987; *accepted* 24 *December* 1987)

Abstract—The metabolism and subsequent immunosuppressive effects of dimethylnitrosamine (DMN) were investigated in mixed cultures of mouse hepatocytes and mouse splenocytes. Hepatocytes were shown to activate DMN to an immunosuppressive form that caused the suppression of the *in vitro* antibody response to the T-dependent antigen, sheep erythrocytes (SRBC). A significant increase in the binding of DMN metabolites to trichloroacetic acid (TCA) precipitable material in splenocytes was induced when 94 μM [^{14}C-*methyl*]DMN was added to the co-culture medium, indicating that reactive intermediates of DMN were transferred from hepatocytes to splenocytes and resulted in alkylation of macromolecules in splenocytes. The amount of [^{14}C]DMN bound to TCA precipitable material in splenocytes increased in a time-dependent manner up to 4 hr of incubation. Aminoacetonitrile (AAN), a high-affinity DMN demethylase inhibitor, reversed the suppression by low concentrations of DMN (0.5 to 5 mM), but not by high concentrations of DMN (>5 mM). AAN also inhibited the binding of [^{14}C]DMN to both hepatocytes and splenocytes. These results suggest that reactive metabolites of DMN are released from hepatocytes and that the suppression of the antibody response by DMN is mediated via these reactive intermediates.

Dimethylnitrosamine has been consistently shown to require metabolic activation in order to exert its biological effects including cytotoxicity, mutagenicity and immunosuppression [1–4]. The key metabolic activation step is believed to be the oxidation of the alpha-hydrocarbon as catalyzed by the cytochrome P-450 monooxygenase system, generally known as dimethylnitrosamine (DMN) demethylase. Such oxidation leads to the unstable alpha-hydroxylmethylnitrosamine which spontaneously breaks down into formaldehyde and a methylcarbonium ion or equivalent species [5–7]. These reactive metabolites can result in DNA alkylation and subsequently produce mutagenic effects *in vivo* and *in vitro* [8, 9].

In light of the relationship between the mutagenicity of DMN and its potency as an alkylator of macromolecules, the immunosuppressive effects of DMN have been investigated. Wayneforth and Magee [10] reported that a single dose of DMN in mice reduced the humoral immune response. Hard [11] has also shown that a single dose of DMN suppressed the antibody response in rats. Interestingly, in an earlier study, Hard demonstrated that a single exposure to DMN did not depress T-lymphocyte functions [12]. More recently, Holsapple *et al.* showed that the repeated exposure to DMN in mice produced significant effects on humoral immunity [4], cell-mediated immunity [13] and tumor susceptibility [14]. Humoral immunity was especially sensitive to DMN. Recently we have begun studying the mechanism of DMN-induced suppression of a humoral immune response *in vivo* and *in vitro*, and have demonstrated that B lymphocytes represent a primary cell type responsible for the suppressed antibody response [15]. The hypothesis we have presumed is that the immunosuppressive effects of DMN are mediated by the reactive intermediates responsible for its mutagenic and carcinogenic effects. We recently developed a co-culture system consisting of primary mouse hepatocytes as a metabolic activation system and mouse splenocytes as target cells [16]. Our initial results demonstrated that the immunosuppression by DMN was hepatocyte dependent and was presumably mediated by reactive metabolites. We have also shown recently that, in animals exposed to [^{14}C]DMN, there is an accumulation of trichloroacetic acid (TCA) precipitable material in macromolecules of lymphocytes [17].

The objective of the present study was to characterize further the relationship between the metabolism of DMN and subsequent suppression of the antibody response by using the co-culture system. Two specific approaches were used to address this objective. First, the distribution of [^{14}C]DMN was determined in the co-culture system. Second, inhibitors of DMN demethylase were tested for their abilities to alter the distribution of DMN metabolites and to ameliorate the DMN-induced immunosuppression. Initially, two different inhibitors of DMN demethylase were tested: aminoacetonitrile

† Address correspondence to: Michael P. Holsapple, Ph.D., Department of Pharmacology and Toxicology, Medical College of Virginia, Richmond, VA 23298.

(AAN; [18]) and diethyldithiocarbamate (DEDTC; [19]). These results are important since they indicate that the *in vitro* immunosuppression by DMN is mediated by reactive intermediates, thereby confirming that the immunosuppressive metabolite has sufficient stability to be able to pass from hepatocytes to splenocytes.

MATERIALS AND METHODS

Animals. Female (C57B1/6 × C3H)F_1 (B6C3F_1) mice were purchased from Litton (Frederick, MD). The mice arrived at 5–6 weeks of age and were quarantined for 1 week prior to use. Animals were randomized, weighed, and housed four per cage. The mice were used as the source of spleen cells when they were 17–20 g in weight and were used for isolation of hepatocytes when they were 20–23 g in weight. The mice were maintained on Purina Lab Chow and tap water *ad lib.* The animal quarters were maintained at 21–24° and 40–60% relative humidity. A 12-hr light/dark cycle was used.

Chemicals and other materials. Chemicals and cell culture materials were obtained from the following sources: [^{14}C-*methyl*]dimethylnitrosamine (DuPont/Neu., Boston, MA); DMN (Aldrich Chemical Co., Milwaukee, WI); AAN, DEDTC and Collagenase type 1 (Sigma Chemical Co., St. Louis, MO); RPMI, Hanks' Balanced Salt Solution (HBSS) and Waymouth medium (Gibco Laboratories, Chagrin Falls, OH); Vitrogen (Collagen Corp., Palo Alto, CA); fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT); sheep erythrocytes (SRBC) (Colorado Serum, Denver, CO); and 6-well cluster plates (Costar, Cambridge, MA).

Isolation and culture of primary mouse hepatocytes. Mouse hepatocytes were isolated using a collagenase perfusion technique as described by Klaunig *et al.* [20] with minor modifications as previously described [16]. In brief, the liver was initially perfused with 50 ml of Ca^{2+}, Mg^{2+}-free HBSS containing 0.05 of mM ethyleneglycolbis(aminoethylether)tetraacetate (EGTA), and then with 100 ml of HBSS containing collagenase (100 units/ml) and trypsin inhibitor (100 units/ml). After washing the cells three times by centrifuging at 50 *g*, they were counted on a hemacytometer and the viability was determined using trypan blue exclusion. The viability was routinely above 80%. The cell suspension was then adjusted to 1×10^6 cells/ml in culture medium containing 5% FCS, and 1 ml was pipetted into individual wells of 6-well cluster plates pre-coated with Vitrogen 100. The culture medium consisted of Waymouth's MB 751/1 medium supplemented with hormones and other chemicals as described by Decad *et al.* [21]. The cultures were incubated at 37° in a humidified CO_2 incubator. After 2 hr of incubation, the medium was changed in order to remove dead or unattached hepatocytes. After 20–24 hr of incubation, the medium was aspirated off and the plates were washed twice with 1 ml of RPMI 1640 medium. The hepatocytes were then used for co-culturing with mouse splenocytes.

Isolation and treatment of splenocytes. Spleen cell suspensions were obtained using the method described by White and Holsapple [22]. The cells were suspended with RPMI 1640 and adjusted to 3×10^7 cells/ml. One milliliter of the splenocyte suspension was added to each of the primary hepatocyte culture wells. Chemicals were then added directly to the cultures. DMN was diluted with RPMI 1640, and AAN and DEDTC were dissolved in phosphate-buffered saline (PBS). The cultures were then incubated for the designated times (1–4 hr) at 37° in the CO_2 incubator without rocking. After incubation, the plates were gently swirled, and splenocytes were harvested with Pasteur pipettes and transformed to sterile plastic tubes. The plates were then washed twice with 1 ml of RPMI 1640. The washings were combined with the splenocyte suspensions and centrifuged at 180 *g* for 10 min. The pellet was washed once with 2 ml of RPMI 1640 and resuspended in RPMI 1640 with 10% fetal calf serum.

In vitro *antibody response.* The antibody response to SRBC was determined as previously described [16]. The spleen cells recovered from the hepatocyte wells were adjusted to 10^7 cells/ml in RPMI containing 10% fetal calf serum. Triplicate cultures for each test group were established in 48-well culture plates (Costar, Cambridge, MA), and each well contained 0.5 ml of cell suspension with 50 μM 2-mercaptoethanol and 5×10^6 SRBC. The cultures were incubated with rocking for 5 days in an atmosphere of 10% CO_2; 7% O_2 and 83% N_2. IgM secreting antibody forming cells (AFC) were enumerated using a modified Jerne plaque assay [23], and viability was assessed by the pronase method as previously described [4]. Results were expressed as AFC/10^6 recovered cells ± SE.

Determination of the binding of DMN to TCA precipitates. For determination of the binding of DMN to TCA precipitates, the cultures were incubated with 5 μCi of [^{14}C]DMN (sp. act. 54.1 mCi/mmol) for the designated times. After incubation, spleen cells were harvested with Pasteur pipettes and transferred to plastic tubes. The plates were washed twice with 1 ml of PBS (0.05 M; pH 7.2). The washings were combined with the splenocyte suspensions. The plates were then incubated with 1 ml of collagenase solution (100 units/m HBSS) at 37° for 10 min. Hepatocytes attached to the collagen-coated wells were detached by this procedure. The contaminating splenocytes were separated from the detached hepatocytes by repeated pipetting and centrifuging three times at 50 *g* for 4 min. The supernatant fractions were combined with the splenocyte suspensions and the hepatocytes remained in the pellet. Hepatocytes were not detected in the splenocyte suspensions by microscopic examination. The cells were then centrifuged at 250 *g* for 10 min. The supernatant fraction from this spin (medium) was removed and sampled. The pellets (splenocytes or hepatocytes) were resuspended in PBS and washed twice by centrifugation and resuspension in PBS. Splenocytes were treated with Tris–NH_4Cl solution in order to remove any residual SRBC. Acid precipitation of cell suspension was achieved by addition of TCA at a final concentration of 5% (w/v) followed by vortexing and centrifugation at 650 *g* for 20 min. The supernatant fractions were removed, and the pellet was washed twice with 5% TCA (w/v) and then dissolved in 1 ml of 0.1 N NaOH. Protein was

determined by the method of Lowry *et al.* [24] with bovine serum albumin as the standard. Radioactivity was determined by liquid scintillation counting in an LKB rackbeta counter using Permafluor V (Packard, Downers Grove, IL) as the scintillation fluid.

Statistical analysis. For the results of all studies, a Dunnett's *t*-test [25] was performed if a one-way analysis of variance of the means showed treatment effects.

RESULTS

Previous results from this laboratory showed that the suppression of the antibody response by DMN is dependent upon rocking speed during the co-culture period and is produced most effectively under the no rocking condition [16]. Therefore, all the experiments in this study were done without rocking. As shown in Fig. 1, DMN was activated to an immunosuppressive form and produced the suppression in a dose range of 0.5 to 100 mM. There was no suppression by DMN without hepatocytes, indicating that splenocytes are not competent in the activation of DMN to immunosuppressive forms, and that DMN requires metabolic activation to produce immunosuppressive effects. The suppression by DMN was demonstrated to be dependent upon both the concentration of DMN (Fig. 1) and the duration of co-culture time (Table 1). As shown in Table 1, the magnitude in the suppression of the response after 3 and 4 hr of incubation at 1 mM DMN were noted to be 40 and 80% respectively. In contrast, the inhibition by 50 mM DMN was 32% even after 1 hr of incubation and was nearly complete after 2 hr of incubation. Therefore, the inhibition by low concentrations of DMN required a longer incubation time.

Studies designed to confirm that reactive intermediates interact with splenocytes were performed with [^{14}C]DMN. Distribution to TCA precipitable material can be interpreted to indicate alkylation of macromolecules by methylating intermediates

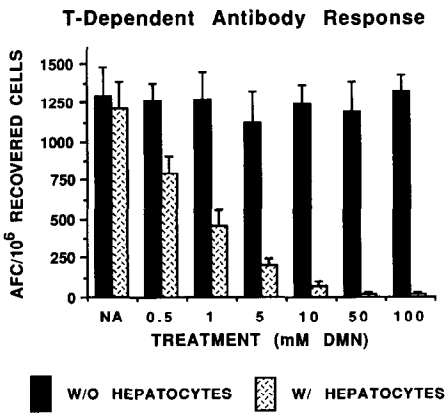

Fig. 1. Dose–response relationship of the suppression of antibody production by dimethylnitrosamine (DMN). Spleen cells were incubated with nothing (naive; NA) or DMN (0.5 to 100 mM) without hepatocytes (dotted bars) or with hepatocytes (hatched bars). Values are means ± SE, N = four replicate wells per treatment.

Table 1. Time course in the suppression of antibody production by DMN

Incubation time (hr)	AFC/10^6 recovered cells 1 mM	50 mM
1	1059 ± 82	785 ± 20**
2	1188 ± 169	33 ± 9**
3	584 ± 48**	3 ± 3**
4	230 ± 29**	3 ± 3**

Control values of AFC/10^6 recovered cells are 1137 ± 93 (naive) and 1149 ± 154 (hepatocytes only). Values represent the mean ± SE from three replicate cultures stimulated with SRBC.

** $P < 0.01$ as determined by ANOVA and Dunnett's *t*-test using the naive group as the control.

[26, 27]. Table 2 shows distribution of [^{14}C]DMN to splenocytes and hepatocytes in a co-culture system after 4 hr of incubation with 94 μM [^{14}C]DMN. The amount of DMN bound to TCA precipitable material in splenocytes co-cultured with hepatocytes was increased markedly when compared to splenocytes cultured alone (without hepatocytes). The binding of [^{14}C]DMN to splenocytes co-cultured with hepatocytes showed a 2.8-fold increase on the basis of cell number and a greater increase (4.0-fold) on the basis of protein content. The alkylation of TCA precipitable material in hepatocytes (cpm/mg protein) was 2.6-fold greater than that in splenocytes, indicating that the alkylation of intracellular nucleophiles is more efficient than that to nucleophiles of the target cells. The metabolism of DMN and the associated binding of DMN to TCA precipitable material showed an incubation time-dependent increase in both hepatocytes and splenocytes up to 4 hr of incubation. As shown in Table 3, when compared to splenocytes alone, 1 hr of incubation resulted in a 38% increase in the binding of DMN to TCA precipitable material in splenocytes cultured with hepatocytes, whereas a 317% increase was demonstrated after 4 hr of incubation in the same group. The mount of DMN associated with hepatocytes was also increased in a time-dependent manner. The results for the 4-hr time point in Table 3 agree nicely with the results in Table 2, indicating the reproducibility of the effect. The last column in Table 3 indicates the total cpm recovered after each incubation. The percentages of total recovered cpm to naive control (medium only; data not shown) after 1-, 2-, 3- and 4-hr co-culture periods were 83.5%, 75.8%, 70.2% and 56.1% respectively. These results indicate that appreciable quantities of the radioactivity derived from [^{14}C]DMN were not recoverable and were suspected to be lost in the gas phase as released formaldehyde.

AAN and DEDTC are known as inhibitors of mixed-function oxygenase enzymes, especially DMN demethylase [18, 19]. Both agents reduce DMN-induced hepatotoxicity *in vivo* [19]. Both AAN and DEDTC were initially used in this study to test whether the DMN-induced suppression of the response to SRBC was mediated via reactive intermediates produced by DMN demethylase. The

Table 2. Distribution of [^{14}C]DMN to splenocytes and hepatocytes

Sample*	cpm/10^7 cells (pmol DMN/10^7 cells) Total	TCA precipitable material	TCA supernatant fraction	cpm/mg protein (pmol DMN/mg protein)
SPLC only	1,357 ± 227† (12.6 ± 2.2)	806 ± 76 (7.6 ± 0.7)	501 ± 114 (4.7 ± 1.1)	1,916 ± 265 (17.8 ± 2.5)
Splenocytes cultured with hepatocytes	3,675 ± 711 (35.2 ± 6.6)	3,003 ± 351 (28.2 ± 3.3)	363 ± 60 (3.4 ± 0.6)	7,704 ± 388 (71.7 ± 3.6)
Hepatocytes	ND‡	ND	ND	19,460 ± 2,558 (182.1 ± 23.8)

* Cultures were incubated with 5 μCi [^{14}C]DMN (sp. act. 54 mCi/mmol) for 4 hr.
† Values are mean cpm ± SE, of triplicate cultures. Values in parentheses are mean pmol DMN ± SE of triplicate cultures.
‡ Not determined.

Table 3. Time course for the labeling of TCA precipitable material in *in vitro* incubations with [^{14}C]DMN

Incubation time* (hr)	cpm/mg protein (pmol DMN/mg protein) Splenocytes (alone)	Splenocytes (+ hepatocytes)	Hepatocytes	Total cpm recovered† (× 10^6)
1	1,936 ± 252‡ (18.0 ± 2.3)	2,683 ± 127 (24.9 ± 1.2)	4,595 ± 527 (42.8 ± 4.9)	5.22 ± 0.14
2	1,831 ± 302 (17.0 ± 2.8)	4,781 ± 218 (44.5 ± 2.0)	8,966 ± 486 (83.7 ± 4.5)	4.37 ± 0.31
3	2,072 ± 352 (19.3 ± 3.3)	6,713 ± 266 (67.2 ± 2.5)	13,680 ± 1,870 (127.2 ± 17.4)	4.05 ± 0.12
4	1,902 ± 265 (17.7 ± 2.5)	7,995 ± 396 (74.4 ± 3.7)	17,920 ± 1,290 (166.8 ± 11.9)	3.28 ± 0.06

* Incubations were done with 5 μCi of [^{14}C]DMN (sp. act. 54 mCi/mmol) for the designated times (1–4 hr).
† Total cpm recovered in medium alone was 6.25 ± 0.24 (× 10^6).
‡ Values are mean cpm ± SE of triplicate cultures. Values in parentheses are mean pmol DMN ± SE of triplicate cultures.

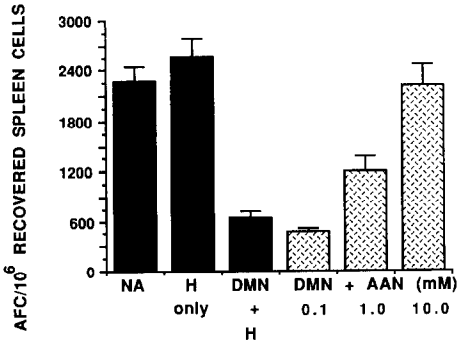

Fig. 2. Dose–response relationship of the reversal of DMN-induced suppression of antibody production by aminoacetonitrile (AAN). Spleen cells were cultured with nothing (naive; NA), hepatocytes only (H only), hepatocytes plus DMN (DMN, 1 mM) or hepatocytes plus DMAN (1 mM) plus AAN (0.1 to 10.0 mM). Values are means ± SE, N = four replicate wells per treatment.

Table 4. Effect of AAN on the labeling of TCA precipitable material by [^{14}C]DMN

AAN (mM)	cpm/mg protein (pmol DMN/mg protein) Splenocytes (+ hepatocytes)	Hepatocytes
0	7,515 ± 497* (70.0 ± 4.6)	18,470 ± 2,078 (171.9 ± 19.3)
1	2,629 ± 78 (25.1 ± 0.7)	6,381 ± 2,097 (59.4 ± 10.2)
10	1,467 ± 102 (13.6 ± 0.9)	4,842 ± 590 (45.1 ± 5.5)

Each sample was incubated with 5 μCi of [^{14}C]DMN (sp. act. 54 mCi/mmol) for 4 hr.
* Values are mean cpm ± SE of triplicate cultures. Values in parentheses are mean pmol DMN ± SE of triplicate cultures.

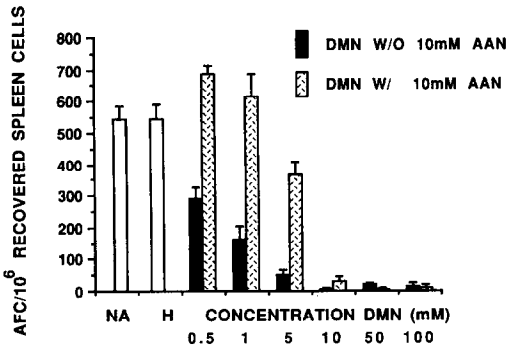

Fig. 3. Effects of the addition of 10.0 mM AAN to the co-culture medium on the suppression of antibody production by DMN. Spleen cells were cultured with nothing (naive; NA; open bar), hepatocytes only (H; open bar) or hepatocytes plus DMN (0.5 to 100 mM) and either AAN (hatched bars) or no AAN (dotted bars). Values are means ± SE, N = four replicate wells per treatment.

results with DEDTC were disappointing since concentrations as low as 10^{-5} M produced a complete suppression of the antibody response to SRBC in the absence of either DMN or hepatocytes (data not shown). These results are consistent with the previously demonstrated profile of activity of DEDTC as an immune modulator [28, 29]. These reports showed that *in vivo* exposure to DEDTC augmented a variety of T-cell mediated functions. The direct effects of DEDTC have heretofore not been investigated extensively. These studies are currently underway. The results with AAN were much more consistent with the predicted effects of blocking DMN demethylase activity. As shown in Fig. 2, AAN reversed the suppression by DMN in a dose-dependent manner when it was added directly to the co-culture medium with 1 mM DMN. The suppression by DMN was not affected by 0.1 mM AAN, was partially reversed by 1.0 mM AAN, and was completely reversed by 10 mM AAN. Antibody responses were not affected by doses of 0.1 to 10 mM AAN (data not shown), indicating that AAN has no direct effects on the function of splenocytes. AAN also decreased the binding of [^{14}C]DMN to both hepatocytes and splenocytes (Table 4). The binding of [^{14}C]DMN was reduced by 74% in hepatocytes plus 10 mM AAN when compared to hepatocytes without AAN. The amount of [^{14}C]DMN bound to splenocytes plus 10 mM AAN was comparably reduced by 80% when compared to splenocytes with no AAN. Figure 3 shows the effects of 10 mM AAN on the suppression by various concentrations of DMN. AAN reversed the suppression by low concentrations of DMN. The suppression by 0.5 and 1 mM was reversed completely, and the suppression by 5 mM was partially reversed (85% compared to control) by AAN. The suppression by concentrations of DMN above 10 mM was not affected.

DISCUSSION

Alkylation of genetic material is generally accepted to be the primary mechanism of the mutagenicity and carcinogenicity of *N*-nitrosamines. There is some controversy concerning the specific metabolic pathway of DMN which generates the ultimate reactive metabolites. It is generally believed that the toxic intermediate, methyldiazonium, which is a final product of alpha-hydroxylation of DMN, causes the alkylation of macromolecules and subsequently induces carcinogenic and/or mutagenic effects [30].

While there are many papers describing the genotoxicity of the nitrosamines, another form of DMN toxicity that has not been well studied is the suppression of acquired immunity. Animals exposed to DMN demonstrate significantly reduced cell-mediated and humoral immune responses after single or multiple exposure [4, 10–14]. *In vitro* treatment with DMN also causes the suppression of humoral immune responses in a co-culture system of murine hepatocytes and splenocytes [16]. The mechanisms for the effects of DMN on immune responses have not been elucidated. Based on the mutagenic property of DMN, we adopted a working hypothesis that DMN-induced immunosuppression would involve reactive intermediates. The objective of the present studies was to investigate our hypothesis by using *in vitro* antibody response to SRBC and a co-culture system of hepatocytes and splenocytes.

In vitro antibody assays have been proposed as models to characterize immunosuppressive chemicals [31] and are a useful tool to investigate the mechanism of immunosuppressive effects. Since spleen cells have limited capability in metabolism, metabolic activation must be provided when studying immunosuppressive effects of chemicals like DMN (i.e. those dependent upon metabolism). Investigations using *in vitro* approaches have indicated that DMN was metabolized to formaldehyde by enzymes of the microsomal fraction of liver [2, 7, 32]. The activation of DMN to a mutagenic form with hepatocytes was 1000-fold greater than that with an S-9 fraction in the V-79 cell mutagenesis system [33]. Rumruen and Pool [34] also reported that intact hepatocytes showed higher activity to convert DMN to a mutagenic form than S-9 fraction in the Ames test. We have shown recently that intact hepatocytes but not liver homogenates (S-9 fractions) could metabolize DMN to an immunosuppressive form [35].

The results in Tables 2 and 3 show that a significant alkylation in hepatocytes was produced by incubation with radiolabeled DMN. The results in Table 3 also indicate that large amounts of activity derived from [^{14}C]DMN were lost in the gas phase, presumably by metabolism to formaldehyde. Total cpm recovered after 4 hr of incubation was reduced by 44%, suggesting that at least 44% of DMN added to culture medium was converted to volatile metabolites. The hepatocytes in this study were used 20–24 hr after isolation, but they were still competent in metabolizing DMN to an immunosuppressive form and gave rise to the alkylation. The significant increase in the alkylation in TCA precipitable material in splenocytes cultured together with hepatocytes and DMN (compared to the splenocytes only) indicates that alkylating intermediates could be transferred from hepatocytes to splenocytes. Our results are consistent with the finding of Umbenhauer and Pegg [36], who showed that

freshly isolated hepatocytes are able to metabolize DMN, and that the intermediate, nitrosohydroxymethylamine, results in alkylation of intracellular and extracellular DNA. Umbenhauer and Pegg [36] reported that cellular alkylation was complete in 2 hr, the time at which the amounts of 7-methylguanine adducts and extracellular DNA alkylation exceeded the intracellular alkylation. In our co-culture system, metabolism was continued up to 4 hr and the alkylation in both hepatocytes and splenocytes increased in a time-dependent manner. Our results demonstrating an apparent transfer of reactive intermediates are also consistent with two previous studies using whole animal exposure to DMN, which showed that radioactivity derived from [^{14}C]DMN is accumulated in spleen cells [17] and nonparenchymal liver cells [37]. Neither spleen cells nor nonparenchymal liver cells are capable of metabolizing DMN. Isolation of radioactivity following exposure to [^{14}C]DMN in TCA precipitable material may be the result of methylation by reactive intermediates, as described above, and of incorporation of ^{14}C via the active C-1 metabolic pool [26, 38]. While our results cannot be used to determine the relative role played by incorporation via the C-1 pool, it is important to emphasize that Barrows [38] demonstrated that only 30% of the methylguanine is produced through C-1 incorporation. To summarize: our results suggest that an alkylating species can be generated within hepatocytes, can diffuse out and be transferred to splenocytes, and can react with macromolecules in splenocytes. The suppression of the antibody response to SRBC may be a consequence of the alteration of function of spleen cells caused by these alkylations. This interpretation is supported by the positive correlation between the time-dependent increase in alkylation (Table 3) and the time-dependent suppression of the antibody response by 1 mM DMN (Table 1). This interpretation is also supported by the induction of mutations in cells which do not metabolize DMN when these cells are cultured together with isolated hepatocytes and DMN [8, 39].

Another approach to demonstrate the role of reactive metabolites in DMN-induced suppression of humoral immunity is to use an inhibitor of DMN demethylase. The measurement of AFC response and alkylation under the influence of an inhibitor is likely to provide useful information on the mechanism of immunosuppressive effects of DMN. AAN is reported to inhibit the metabolism of DMN [18, 40] and the induction of liver DNA strand breaks *in vivo* [19]. The results in Figs. 2 and 3 and Table 4 showed that the addition of AAN into the co-culture medium reversed the suppression by DMN and prevented the alkylation in both hepatocytes and splenocytes. The results suggested a positive correlation between the magnitude of the decrease in alkylation and the degree of reversal of DMN-induced immunosuppression. The magnitude of reversal of DMN-induced suppression by AAN was different depending upon concentrations of DMN (Fig. 3). The suppression by low concentrations of DMN (0.5 to 5 mM) was reversed by the addition of AAN, whereas the suppression of higher concentrations of DMN was not affected by AAN. Many investigations have indicated that a multiple enzyme pathway may be involved in the metabolism of DMN [32, 41]. Arcos *et al.* [42] have reported the existence of two kinetically distinct DMN demethylase enzymes. High-affinity DMN demethylase was defined as the isoenzyme active in the substrate range of 0.8 mM, while low-affinity DMN demethylase was the isoenzyme responsible for the metabolism of DMN at concentrations of 8–20 mM. Therefore, AAN seems to selectively affect high-affinity DMN demethylase in view of our results.

We have used two different approaches to investigate the role of reactive metabolites in the suppression of *in vitro* antibody response to SRBC by DMN. A significant increase in the alkylation in splenocytes was seen when splenocytes were cocultured with hepatocytes. The time-dependent suppression by DMN was correlated with the time-dependent increase in amounts of alkylation in splenocytes. The suppression by DMN was reversed by AAN, which also resulted in a reduction in the amount of alkylation. Our results suggest that the immunotoxicity of DMN is mediated by reactive intermediates released from hepatocytes that alter lymphocyte function as a consequence of alkylation of macromolecules.

Acknowledgements—The authors wish to thank Ms. Brenda Rexrode and Mrs. Sheryol Cox for the preparation of this manuscript. This work was supported by NIH Grant 1R01ES03564, and by a Korea Science and Engineering Foundation Research Grant.

REFERENCES

1. Magee PN and Barnes JM, Carcinogenic nitroso compounds. *Adv Cancer Res* **10**: 163–246 (1967).
2. Heath DF, The decomposition and toxicity of dialkylnitrosamines in rats. *Biochem J* **85**: 72–91 (1962).
3. Druckrey H, Preussman R, Ivankovic S and Schmal D, Organotrope carcinogene wirkungen bei 65 verchiedenen *N*-nitrosoverbindungen an BD-ratten. *Z Krebsforsch* **69**: 103–201 (1967).
4. Holsapple MP, Tucker AN, McNerney PJ and White KL, Effects of *N*-nitrosodimethylamine on humoral immunity. *J Pharmacol Exp Ther* **229**: 493–500 (1984).
5. Milstein S and Guttenplan JB, Near quantitative production of molecular nitrogen from metabolism of dimethylnitrosamine. *Biochem Biophys Res Commun* **87**: 338–342 (1979).
6. Michejda CJ, Kroeger-Koepke MB, Koepke SR, Magee PN and Chu C, Nitrogen formation during *in vivo* and *in vitro* metabolism of *N*-nitrosamines. In: *Banbury Report 12: Nitrosamines and Human Cancer* (Ed. Magee PN) pp. 69–85. Cold Spring Harbor Laboratory, New York (1982).
7. Lai PY and Arcos TC, Dialkylnitrosamine bioactivation and carcinogenesis. *Life Sci* **27**: 2149–2165 (1980).
8. Jones CA and Huberman E, A sensitive hepatocyte mediated assay for the metabolism of nitrosamines to mutagens for mammalian cells. *Cancer Res* **40**: 406–411 (1980).
9. Yoo JSH and Yang CS, Enzyme specificity in the metabolic activation of *N*-nitrosodimethylamine to a mutagen for Chinese hamster V-79 cells. *Cancer Res* **48**: 5569–5574 (1985).

10. Wayneforth HB and Magee PN, The effect of *N*-nitro-*N*-nitrosomethylurea and *N*-dimethylnitrosamine on cell-mediated and humoral immune responses in rats and mice. *Br J Cancer* **30**: 512–523 (1974).
11. Hard GC, Effect of a single carcinogenic dose of dimethylnitrosamine on antibody responses in the rat. *Oncology* **38**: 47–52 (1981).
12. Hard GC, Thymus-dependent lymphocyte function in dimethylnitrosamine-induced renal carcinogenesis is not depressed. *Oncology* **31**: 139–146 (1975).
13. Holsapple MP, Bick PH and Duke SS, Effects of *N*-nitrosodimethylamine on cell-mediated immunity. *J Leukocyte Biol* **37**: 367–381 (1985).
14. Duke SS, Schook LB and Holsapple MP, Effects of *N*-nitrosodimethylamine on tumor susceptibility. *J Leukocyte Biol* **37**: 383–394 (1985).
15. Johnson KW, Munson AE and Holsapple MP, Primary cellular target responsible for dimethylnitrosamine-induced immunosuppression in the mouse. *Immunopharmacology* **13**: 47–60 (1987).
16. Kim DH, Yang KH, Johnson KW and Holsapple MP, Suppression of *in vitro* antibody production by dimethylnitrosamine in mixed cultures of mouse primary hepatocytes and mouse splenocytes. *Toxicol Appl Pharmacol* **87**: 32–42 (1987).
17. Johnson KW, Munson AE, Kim DH and Holsapple MP, The role of reactive metabolites in the immunosuppression by *N*-nitrosodimethylamine. *J Pharmacol Exp. Ther* **240**: 847–855 (1987).
18. Fiume L, Campadelli-Fiume G, Magee PN and Holsman J, Cellular injury and carcinogenesis: Inhibition of metabolism of dimethylnitrosamine by aminoacetonitrile. *Biochem J* **120**: 601–605 (1970).
19. Abanobi SE, Popp JA, Chang SK, Harrington GW, Lotlikar PD, Hadjiolov D, Levitt M, Rajalakshmi S and Sarma DSR, Inhibition of dimethylnitrosamine-induced strand breaks in liver DNA and liver cell necrosis by diethyldithiocarbamate. *J Natl Cancer Inst* **58**: 263–271 (1977).
20. Klaunig JE, Goldblatt PJ, Hinton PE, Lipsky MM, Chacko J and Trump BF, Mouse liver cell culture: I. Hepatocyte isolation. *In Vitro* **17**: 913–925 (1981).
21. Decad GM, Hsieh PPM and Byard JL, Maintenance of cytochrome P-450 and metabolism of aflatoxin B in primary hepatocyte cultures. *Biochem Biophys Res Commun* **78**: 279–287 (1977).
22. White KL Jr. and Holsapple MP, Direct suppression of *in vitro* antibody production by mouse spleen cells by the carcinogen benzapyrene but not by the non-carcinogenic congener benzepyrene. *Cancer Res* **44**: 3388–3393 (1984).
23. Bullock RW and Moller E, Spontaneous B cell activation due to loss of normal mouse serum suppressor. *Eur J Immunol* **2**: 514–517 (1972).
24. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the folin phenol reagent. *J Biol Chem* **193**: 265–275 (1951).
25. Dunnett CW, A multiple comparison procedure for comparing several treatments with a control. *J Am Stat Assoc* **50**: 1096–1121 (1955).
26. Chin AE, and Bosmann HB, Hepatic microsomal metabolism of *N*-nitrosodimethylamine to its methylating agent. *Toxicol Appl Pharmacol* **54**: 70–89 (1980).
27. Grilli S, Bragaglia RB and Prodi G, An *in vitro* study on the interaction between dimethylnitrosamine and nucleic acids via a microsomal system. *Gann* **68**: 129–137 (1977).
28. Renoux G and Renoux M, Immunopotentiation and anabolism induced by sodium diethyldithiocarbamate. *J Immunopharmacol* **1**: 247–267 (1979).
29. Renoux G and Renoux M, Thymus-like activities of sulphur derivatives on T-cell differentiation. *J Exp Med* **145**: 466–471 (1977).
30. Dipple A, Michejda CJ and Weisberger EK, Metabolism of chemical carcinogens. *Pharmacol Rev* **22**: 265–296 (1985).
31. Archer DL, Smith BG and Bukovic-Wess JA, Use of an *in vitro* antibody-producing system for recognizing potentially immunosuppressive compounds. *Int Arch Allergy Appl Immunol* **56**: 90–93 (1978).
32. Lake BG, Phillips JC, Heading CE and Gangolli SD, Studies on the *in vitro* metabolism of dimethylnitrosamine by rat liver. *Toxicology* **5**: 297–309 (1978).
33. Langenbach R and Oglesby L, The use of intact cellular activation systems in genetic toxicology assays. In: *Chemical Mutagens* (Ed. de Serres F), Vol. 8, pp. 55–93. Plenum Press, New York (1986).
34. Rumruen K and Pool BL, Metabolic activation capabilities of S9 and hepatocytes from uninduced rats to convert carcinogenic *N*-nitrosamines to mutagens. *Mutat Res* **140**: 147–153 (1984).
35. Johnson KW, Kim DH, Munson AE and Holsapple MP, Dependence on intact cells of the *in vitro* activation of dimethylnitrosamine to an immunosuppressive form. *Mutat Res* **182**: 211–221 (1987).
36. Umbenhauer DR and Pegg AE, Alkylation of intracellular and extracellular DNA by dimethylnitrosamine following activation by isolated rat hepatocytes. *Cancer Res* **41**: 3471–3474 (1981).
37. Planche-Martel G, Likhachev A, Wild CP and Montesano R, Modulation of repair of 8-methylguanine in parenchymal and nonparenchymal liver cells of rats treated with dimethylnitrosamine. *Cancer Res* **45**: 4768–4773 (1985).
38. Barrows LR, Methylation of DNA guanine via the 1-carbon pool in dimethylnitrosamine-treated rats. *Mutat Res* **173**: 73–79 (1986).
39. Langenbach R, Freed HJ and Huberman E, Liver cell-mediated mutagenesis of mammalian cells by liver carcinogens. *Proc Natl Acad Sci USA* **75**: 2864–2867 (1978).
40. Lake BG, Harris RA, Collins MA, Cottrell RC, Phillips JC and Gangolli SC, Studies on the metabolism of dimethylnitrosamine *in vitro* by rat liver preparations. II. Inhibition by substrates and inhibitors of monoamine oxidase. *Xenobiotica* **12**: 567–579 (1982).
41. Kroeger-Koepke MB and Michejda CJ, Evidence for several demethylase enzymes in the oxidation of dimethylnitrosamine and phenylmethyl-nitrosamine by rat liver fractions. *Cancer Res* **39**: 1587–1591 (1979).
42. Arcos JC, Davies DI, Brown CEL and Arcos MF, Repressible and inducible enzyme forms of dimethylnitrosamine-demethylase. *Z Krebsforsch* **89**: 181–199 (1977).