Suppressive Effects of Caffeine on the Immune Response of the Mouse to Sheep Erythrocytes[1] (37651)

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(Introduced by Wayburn S. Jeter)
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The existence of naturally occurring chemical or biological agents capable of expressing an immunosuppressive effect is an important consideration. Most of the investigations on chemical immunosuppressants have been concerned with purine analogs, alkylating agents, folic acid inhibitors, and other drugs that might have possible clinical application. The present report is concerned with the effect of caffeine on antibody production.

Caffeine, a methylated xanthine, occurs commonly in many consumable products including beverages and medicines. In addition, the effect of caffeine on metabolic function of mammalian tissues and cells suggests that it is a potent inhibitor of enzyme function (1, 2). DNA polymerase activity and the action of magnesium-dependent phosphodiesterase in mammalian cell lines have been found to be inhibited by caffeine. Previous studies in our laboratory suggested that caffeine inhibited the synthesis of antibody *in vitro* (3).

Materials and Methods. Animals and immunization. Adult Swiss Webster mice were injected intraperitoneally with a single 0.5 ml dose of 5% sheep erythrocyte suspension for *in vitro* studies. The animals were sacrificed four days after injection and their spleens removed aseptically.

Hemolysin production and assay. The culturing of spleen cells for hemolysin production and the hemolysin assay procedures for screening of immunosuppressants were previously described (3). Briefly, spleen cells were washed and suspended in phenol red–free Eagle's basal medium containing 3% agamma calf serum (Grand Island Biological Co.) supplement with antibiotics. Four ml of this suspension, containing 1×10^8 cells, were placed in each of several petri dishes and incubated in an atmosphere of 95% air and 5% CO_2 at 37°. After incubation, cells and culture fluids were separated and 1 ml of the supernatant fluid was assayed for hemolysin by adding a standardized erythrocyte suspension which was prepared so that an optical density of 0.7 at 541 nm was obtained when the erythrocytes were completely lysed. The culture fluid–erythrocyte mixture was then incubated at 37° for 30 min with occasional shaking. After this sensitization period, guinea pig complement and veronal buffer were added to all samples except the control, which received only veronal buffer. The mixtures then were incubated at 37° for 1 hr with occasional agitation. After incubation the unlysed cells were removed by centrifugation and the OD of the sample was measured at 541 nm and corrected for any nonspecific lysis of the hemolysin–erythrocyte control. The quantity of hemolysin was expressed as hemolytic units per milliliter (HU/ml) by assigning a value of one to that amount of hemolysin which gave an OD reading of 0.4 at 541 nm.

Preparation of spleen cell cultures for DNA analysis. Cultures containing 1×10^8 cells and 10 μCi/ml of tritiated thymidine (SA = 12.5 mCi/mmole) were incubated at 37°. Caffeine (Calbiochem.) was added at 3 hr to give a concentration of 37 μmole.

Preparation of spleen cultures for RNA analysis. Beginning at zero time, cultures containing 1×10^8 cells were pulse labelled at

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3-hr intervals for 12 hr and at 6-hr intervals for an additional 12 hr with 10 μCi/ml of ^{14}C-uridine (SA 21.7 mCi/mmole). Caffeine and actinomycin D were added separately to give final concentrations at 37 μM and 3.2 $\times 10^{-9}$ M respectively.

Preparation of spleen cell cultures for protein analysis. Cultures containing 1 $\times 10^8$ cells and 1.0 μCi/ml ^{14}C-isoleucine (SA 12.2 mCi/mmole) were incubated. Final concentrations of 37 μM caffeine and 4.2 $\times 10^{-7}$ M puromycin were obtained by adding the chemicals separately at 3 hr.

DNA and RNA analysis of spleen cell cultures. At appropriate times the cells were harvested by centrifugation. Cell pellets were prepared for liquid scintillation counting according to the procedures described in Bach and Voynow (4). Briefly, the pellets were suspended in 5% trichloroacetic acid (TCA) and the resulting precipitates were dissolved in 0.1 M NaOH and reprecipitated with 6.7% TCA. The digestion process was repeated and the final TCA precipitates were dissolved in Nuclear Chicago solubilizer (Nuclear Chicago Corp., Chicago, Ill.) and suspended in 2,5-bis-5′-*t*-butylbenzoxazolyl-2′-thiophene (BBOT) scintillation mixture. All samples were counted for 10 min on a Packard Tri-Carb Scintillation Counter and the amount of incorporated radioactivity expressed as cpm at 60% machine counting efficiency for tritium and 90% machine counting efficiency for ^{14}C.

Protein analysis of spleen cell cultures. Cultures were removed from incubation at appropriate times and centrifuged. The cell pellets were dialyzed for 5 days against six changes of 0.015 M phosphate buffer, pH 7.6 at 4°. After dialysis the samples were lyophilized and dissolved in distilled water. The samples were prepared as described previously and the radioactivity counted.

Treatment of mice with caffeine. Mice weighing between 25–30 g, were treated with caffeine either prior to or following immunization. Mice treated prior to immunization were injected ip on days —4 and —2 with 200 mg/kg body weight. Mice treated following immunization were injected ip with various concentrations of caffeine (50–200 mg/kg body weight) on day 0 (1 hr after immunization) and on day 2, control animals for both groups were treated according to the same schedules with saline. On day 0 all animals were immunized by ip injection of 0.5 ml of a 5% sheep erythrocyte suspension. The animals were sacrificed 4 days after immunization by cardiac bleeding. The serum was assayed for antibody by microtiter hemagglutination procedures. In selected cases the spleens were aseptically removed immediately following cardiac bleeding, and the cells were prepared and cultured as described above.

Results. Effect of caffeine on hemolysin production in vitro. In the presence of caffeine, hemolysin production was suppressed (Fig. 1). The degree of suppression was dependent on the dose and was observed in cultures containing 1.9–47 μM caffeine/10^8 cells with the inhibition ranging from 12–70%. Caffeine at a concentration of 37 $\mu M/10^8$ cells gave approximately 50% suppression of antibody production; therefore this concentration of caffeine was used in all subsequent *in vitro* experiments. No difference in viability was found between caffeine treated and control cultures by trypan blue exclusion.

Effect of caffeine on DNA synthesis in vitro. DNA synthesis in the presence and absence of caffeine was measured by the incorporation of tritiated thymidine (^{3}H-TdR) into acid insoluble cell fraction. The results of this experiment and the effect of caffeine

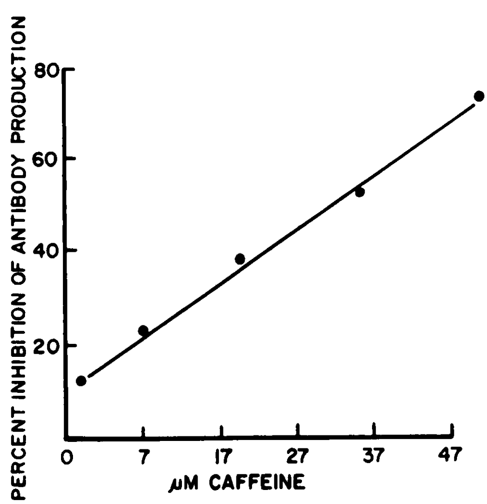

FIG. 1. The effect of increasing concentrations of caffeine on antibody production *in vitro.*

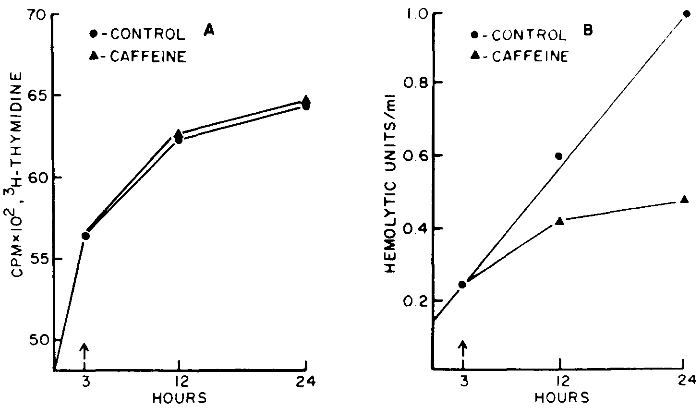

FIG. 2. The effect of caffeine (37 μM) on DNA synthesis (A) and hemolysin production (B) *in vitro*. The arrows indicate the time of caffeine addition to test cultures.

on hemolysin production are presented in Fig. 2. No apparent difference was found in the incorporation of ^{3}H-TdR into DNA between the caffeine treated and untreated cultures (Fig. 2A). However, the presence of caffeine did inhibit the production of hemolysin (Fig. 2B). These results suggest that the suppression of hemolysin production is not dependent on the inhibition of DNA synthesis under these conditions.

Effect of caffeine on RNA synthesis in vitro. The incorporation of ^{14}C-uridine (^{14}C-Urd) into the acid-insoluble cell fraction was used to measure RNA synthesis. The results in Fig. 3A suggest that caffeine had no effect on the incorporation of ^{14}C-Urd into RNA, whereas in the presence of actinomycin D, a known inhibitor of DNA-dependent RNA synthesis, marked suppression of ^{14}C-Urd incorporation occurred. Figure 3B shows that hemolysin production in caffeine-treated cultures was suppressed shortly after its addition, while the effect of actinomycin D was not apparent until later. Although these results indicate that RNA synthesis is required, the suppressive effect of caffeine on hemolysin production appears not to be caused by an inhibition of DNA-dependent RNA synthesis.

Effect of caffeine on protein synthesis in vitro. Protein synthesis in presence and absence of caffeine was measured by the incorporation of ^{14}C-isoleucine (^{14}C-ile) into cell-associated protein. The incorporation of ^{14}C-

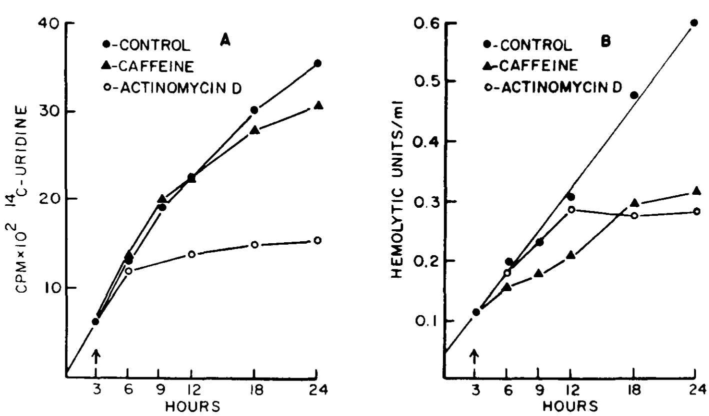

FIG. 3. The effect of caffeine (37 μM) and actinomycin D (3.2×10^{-9} M) on RNA synthesis (A) and hemolysin production (B) *in vito*. The arrows indicate when both chemicals were added to the test cultures.

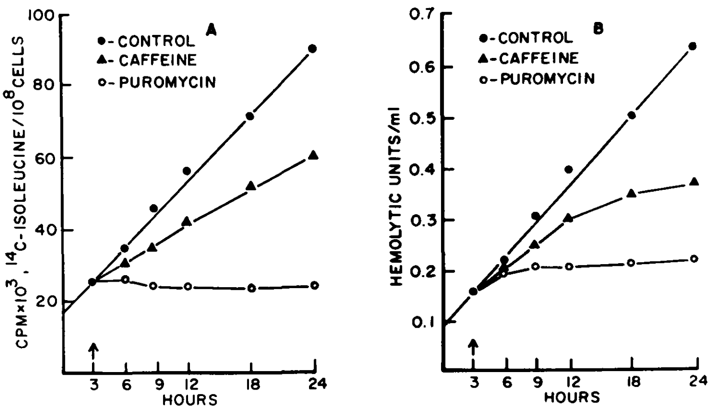

FIG. 4. The effect of caffeine (37 μM) and puromycin (4.2×10^{-7} M) on protein synthesis (A) and hemolysin production (B) *in vitro*. The arrows indicate when both chemicals were added to the test cultures.

ile into protein was suppressed by caffeine but not to the same degree as by puromycin, a known inhibitor of protein synthesis (Fig. 4A). The effect of caffeine and puromycin on hemolysin production is shown in Fig. 4B. An inhibitor effect was apparent shortly after their addition to the cultures. However, it should be noted that at concentrations used the effect of caffeine on hemolysin and total protein synthesis differed from that of puromycin. The effect of puromycin was complete following the expression of its action, while the action of caffeine reduced the rate of total protein and hemolysin synthesis. These results suggest that the suppressive effect of caffeine was due to its ability to act as an inhibitor of protein synthesis.

Effect of caffeine on hemolysin production in vivo. Figure 5A shows the effect of caffeine on antibody production in mice, when the caffeine treatment was given following injection of sheep erythrocytes. Statistically significant differences in serum hemagglutination titers, at 95% confidence limits, were observed when the concentration of caffeine administered was 200 mg (943.4 μM)/kg body weight. However, some individual animals in groups receiving lower doses of caffeine showed depressed titers. Figure 5B shows the results of treatment of mice with 200 mg/caffeine/kg body weight prior to injection of sheep erythrocytes. Once again a statistically significant difference in serum hemagglutination titers at 95% confidence limits is noted.

Evidence correlating *in vitro* and *in vivo* inhibition was obtained by comparing the hemolysin production of spleen cells (10^8)

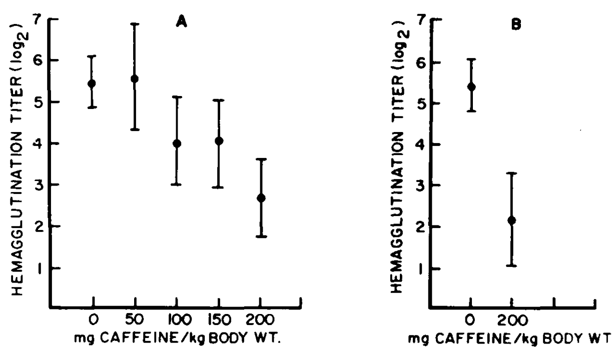

FIG. 5. The effect of caffeine administrated after immunization, day 0, and day 2, (A), and prior to immunization, day 4 and day 2, (B), on antibody production *in vivo*. Each bracket represents the 95% confidence limits.

from caffeine-treated and control mice. Cultures of spleen cells from animals treated with caffeine following injection of sheep erythrocytes showed a 45% reduction in the amount of hemolysin produced *in vitro,* when compared to cultures from control animals. This indicates that the reduced serum hemagglutination titer was due either to a decrease in number of antibody-producing cells or a decrease in the ability of the immunologically competent cells to produce antibody.

Discussion. The results of the above study demonstrate the ability of the methylated xanthine derivative, caffeine, to depress antibody synthesis *in vitro.* The inhibition was directly dependent upon the concentration of caffeine present in the culture fluid. The general mechanism of action appears to be due to the suppression of protein synthesis. However, the exact means by which caffeine suppresses protein synthesis was not determined by this study, but was probably due to multiple effects.

The two effects of puromycin on protein synthesis are considered to be the release of incomplete polypeptides from the polyribosomes and the dissociations of polyribosomes to monoribosomes when active protein synthesis is taking place (5). However, in mice, puromycin has been shown to have a glycogenolytic effect which was associated with an inhibition of the breakdown of CAMP (6, 7). Caffeine has been reported to also inhibit the breakdown of CAMP (2). However, the results using caffeine and puromycin (Fig. 4) suggest basic differences in the action of these chemicals. Consequently, it would be speculative to suggest that the inhibition of protein synthesis by caffeine was due to inhibition of CAMP breakdown.

Recently, theophylline and caffeine were found to enhance the stimulatory effect of synthetic polynucleotides (poly A:U and I:C) on the appearance of antibody-producing spleen cells when concurrently administered *in vivo* (8, 9). In the absence of Poly A:U, theophylline stimulated an increase in the number of antibody-producing cells only when given after antigenic stimulation. Higher concentrations of either theophylline or caffeine reduced the number of antibody-producing cells, if treatment was given at the time of antigenic stimulation. The effect of these synthetic polynucleotides, theophylline and caffeine, supported their conclusion that activation and regulation of immunocompetent cells is dependent on CAMP-mediated events.

In vivo, caffeine suppressed antibody production when given before and after antigenic stimulation at dose of 200 mg/kg body weight (Fig. 5). These results established a correlation with the *in vitro* effect of caffeine.

The data raise the question of whether or not caffeine has any effect upon humans who daily consume large quantities of this substance. Caffeine normally occurs in concentrations of 150–250 mg/8 oz. serving of coffee, tea, and cola flavored drinks. Medicinal preparations also contain concentrations which fall within this range. The experiments of Goldstein, Warren, and Kaizer (10) suggest that adult males had levels of 4.4–6.0 μg caffeine/ml of serum after ingesting 300 mg of caffeine. The absorption of caffeine was considered to be complete within 1 hr. The half-life of caffeine in humans has been reported at 3.5 hr (11). This information suggests that the levels of caffeine required to suppress antibody synthesis are not normally obtained. However, individual differences in sensitivity to its action and the effect of prolonged exposure to low levels of this compound have not been investigated.

Summary. The effects of caffeine on the primary immune response of the mouse to sheep erythrocyte were investigated. The addition of caffeine to cultures of spleen cells from mice 4 days after antigenic stimulation showed depressed antibody production. Caffeine in concentrations of 1.9–47 μM gave dose-dependent suppression ranging from 12–72%. Kinetic studies indicated that caffeine (37 μM) depressed antibody production and cell-associated protein synthesis at approximately the same extent. The synthesis of DNA and RNA was not inhibited under these conditions. Synthesis of DNA-dependent RNA and protein could be inhibited by the addition of actinomycin D and puromycin, respectively, to the culture media. When tested *in vivo* caffeine at a dose of 200

mg/kg body weight given either before or after antigenic stimulation depressed serum antibody levels. The results suggest that caffeine exerts its immunosuppressive effects by depressing protein synthesis, possibly by inhibiting enzymes involved with protein production.

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