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Identification of Suppressive Components in "*Haemophilus somnus*" Fractions Which Inhibit Bovine Polymorphonuclear Leukocyte Function

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"*Haemophilus somnus*" fractions which inhibited iodination of protein by bovine polymorphonuclear leukocytes were isolated by heat extracting a washed bacterial suspension at 60°C or incubating the bacterial suspension at 37°C and were partially purified by ultrafiltration. The components in each fraction were separated by reverse-phase high-performance liquid chromatography and identified as ribonucleotides, a ribonucleoside, and purine and pyrimidine bases. Most of the compounds were found to be inhibitory to iodination in a dose-dependent manner. When the effect of each component on iodination at the concentrations present in "*H. somnus*" fractions was determined, it was found that guanine and GMP were the components responsible for most of the suppression in the fraction isolated by heat extraction, whereas guanine and adenine were the major inhibitory components in the fraction isolated by incubation at 37°C.

The mechanism(s) whereby "*Haemophilus somnus*" overcomes host resistance and induces pathologic change is unknown. Stephens et al. (11) observed increased antibody titers to "*H. somnus*" in acute-phase serum from 16 cattle that died from experimentally induced thromboembolic meningoencephalitis. They suggested that the occurrence of bacteremia in the face of a high serum antibody titer indicated that the phagocytic system of the host was not functioning effectively. More recently, in vitro study of interaction between bovine neutrophils and "*H. somnus*" indicated that ingested "*H. somnus*" was resistant to killing by neutrophils, whereas opsonized *Escherichia coli* and *Staphylococcus epidermidis* were readily killed (3). We have previously demonstrated that live "*H. somnus*" and subcellular fractions of "*H. somnus*" suppress *Staphylococcus aureus* ingestion and iodination but not Nitro Blue Tetrazolium reduction by bovine polymorphonuclear leukocytes (PMNs) (R. D. Hubbard, M. L. Kaeberle, J. A. Roth, and Y.-W. Chiang, Vet. Microbiol., in press). One of the subcellular fractions, which can be retained by an ultrafiltration membrane with a molecular-weight cutoff of 300,000, suppresses both iodination and *S. aureus* ingestion. Another subcellular fraction, which suppresses iodination only, can pass through an ultrafiltration membrane with a molecular-weight cutoff of 1,000. The possible role of the latter bacterial fraction as a virulence factor is indicated by the following facts: (i) PMNs are important in the control of bacterial infections; (ii) degranulation, for which iodination is an indirect measurement, is an important antibacterial mechanism used by PMNs; and (iii) the host would not normally produce antibody against these low-molecular-weight compounds.

In the experimentation reported here, we identified the suppressive components in the low-molecular-weight fraction and also demonstrated that the suppressive components can be released into the fluid phase by the bacterium. The identification of the suppressive components will facilitate the investigation into their potential role as virulence factors and their mechanism(s) of action.

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MATERIALS AND METHODS

Chemicals and reagents. Brain heart infusion (BHI) broth, yeast extract, and Bacto-Agar were purchased from Difco Laboratories, Detroit, Mich. ^{125}I (carrier free in 0.1 M NaOH) and [^{125}I]iododeoxyuridine ([^{125}I]UdR) were purchased from Amersham Corp., Arlington Heights, Ill. Earle's balanced salt solution was purchased from GIBCO Laboratories, Grand Island, N.Y. Zymosan A, lysostaphin, tetrabutylammonium phosphate, AMP (free acid), ADP, adenosine, adenine, GMP (free acid), GDP, guanosine, guanine, CMP (free acid), CDP, cytosine, UMP (free acid), uracil, 5'-nucleotidase (EC 3.1.3.5), and guanase (EC 3.5.4.3) were purchased from Sigma Chemical Company, St. Louis, Mo. Stock solutions (10 mM) of each ribonucleotide, ribonucleoside, purine base, and pyrimidine base were prepared in phosphate-buffered saline (PBS) solution (pH 7.2) except guanine which was dissolved in H_2O and adjusted to pH 12.5 with NaOH. The stock solution of each standard was then diluted in PBS to the desired concentration used in the iodination assay.

Propagation of bacteria. "*H. somnus*" 8025 stored in egg yolk culture at −70°C was inoculated into BHI broth supplemented with 0.5% yeast extract and 5% normal bovine serum (BHISY medium). The broth culture was incubated for 18 to 24 h in a humidified 5% CO_2 incubator and then used to inoculate Roux flasks containing BHISY medium and 2.5% Bacto-Agar. After incubation for 24 h, the bacteria were harvested, washed two times, and suspended in PBS (10 ml/g of cells [wet weight]). A strain of *E. coli* previously shown to be susceptible to killing by bovine neutrophils (3) was inoculated into BHI broth, incubated at 37°C for 18 h, and then used to inoculate Roux flasks containing BHI broth and 2.5% Bacto-Agar. The Roux flasks seeded with *E. coli* were incubated at 37°C for 18 h before the bacteria were harvested, washed, and suspended in PBS.

Preparation of bacterial fractions. The washed "*H.*

somnus" suspension (1.9×10^9 cells per ml) was heat extracted at 60°C for 1 h followed by centrifugation at 27,000 × *g* for 20 min at 4°C. The supernatant was collected and centrifuged at 100,000 × *g* for 3 h at 4°C. The supernatant from the ultracentrifugation was usually stored at −20°C overnight before ultrafiltration. The ultrafiltration was conducted first with a membrane filter with a molecular-weight cutoff of 300,000 (XM300; Amicon Corp., Lexington, Mass.) and then with a membrane filter with a molecular-weight cutoff of 1,000 (Amicon). The final filtrate (designated HE1F-60) was lyophilized and suspended in H_2O to the concentrations desired.

A second low-molecular-weight preparation was isolated from "*H. somnus*" by incubating the washed bacterial suspension in PBS (3.5×10^9 cells per ml) at 37°C for 2 h in a shaking water bath and processing as described above for the 60°C heat extract. This low-molecular-weight fraction was designated HE1F-37. A similar fraction was produced from *E. coli* in an identical manner.

HPLC. The high-performance liquid chromatography (HPLC) system used throughout this study was equipped with a C_{18} column (μBondapak, 7.8 by 300 mm; Waters Associates, Inc., Milford, Mass.) and a guard column (Spheri-10 RP-18 MPLC guard cartridge; Brownlee Labs, Inc., Santa Clara, Calif.). The wavelength of the detector was 277 nm.

PMN preparation. PMNs were prepared from peripheral blood of healthy cattle as previously described (10). Briefly, peripheral blood was collected in an acid-citrate-dextrose solution and centrifuged, and the plasma and buffy coat layers were discarded. Erythrocytes in the packed cell fraction were lysed by brief exposure to hypotonic conditions, and the PMNs in the lysate were pelleted by centrifugation. The residual erythrocytes in the PMN fraction were lysed by a second exposure to hypotonic conditions, and the PMNs were washed and suspended in PBS to a concentration of 5.0×10^7 PMNs per ml. Differential stains indicated that these preparations contained greater than 90% neutrophils.

PMN function tests. The procedures used for evaluating PMN function have been described in detail previously (10). All PMN function tests were conducted in duplicate, and the average of duplicate values was used for calculations.

(i) Iodination. The iodination tests were conducted in polyethylene snap cap tubes (12 by 75 mm; no. 2054; Becton Dickinson Labware, Oxnard, Calif.). The standard reaction mixture contained 2.5×10^6 PMNs, 0.05 μCi of $^{125}I^-$, 40 nmol of NaI, 0.05 ml of opsonized zymosan (10 mg/ml), and 0.3 ml of Earle's balanced salt solution. To determine the effect of "*H. somnus*" fractions on iodination by PMNs, 0.1 ml of bacterial fractions or 0.1 ml of PBS as a control was added to the standard reaction mixture. The reaction was allowed to proceed for 20 min at 37°C, and the amount of trichloroacetic acid-precipitable radioactivity was determined. The results were expressed as nanomoles of NaI per 10^7 PMNs per hour.

(ii) *S. aureus* ingestion. Heat-killed [^{125}I]UdR-labeled *S. aureus* was used to evaluate ingestion by PMNs. The test was conducted in plastic tubes (12 by 75 mm), and the standard reaction mixture contained 0.1 ml of [^{125}I]UdR-labeled *S. aureus*, 0.05 ml of PMNs (2.5×10^6 PMNs; bacteria/PMN ratio, 60:1), 0.05 ml of bovine anti-*S. aureus* serum, and 0.3 ml of Earle's balanced salt solution. To determine the effect of "*H. somnus*" fractions on ingestion by PMNs, 0.1 ml of "*H. somnus*" fractions or 0.1 ml of PBS as a control was added to the standard reaction mixture. The reaction was allowed to proceed for 10 min at 37°C, and the extracellular *S. aureus* was removed by lysostaphin treatment. The PMNs were washed by centrifugation, and the amount of PMN-associated radioactivity was determined. The results were expressed as the percentage of [^{125}I]UdR-labeled *S. aureus* that was ingested.

Data analysis. For the presentation of data, all treatment values were expressed as percentages of the values for the untreated controls. An analysis of variance was used to determine the significance of the differences between the treatment values and those for the untreated controls. The analysis of variance was based on the actual values rather than the percentage of the control values.

RESULTS

Biological activity of HE1F-60 and HE1F-37. Both HE1F-60 and HE1F-37 significantly inhibited iodination by PMNs ($P < 0.001$), whereas neither had an inhibitory effect on ingestion of *S. aureus* (Table 1). The mean iodination value of control PMNs was 28.2 ± 2.3 nmole of NaI per 10^7 PMNs per h (mean ± standard error [SE], $n = 6$), and the mean percentage of [^{125}I]UdR-labeled *S. aureus* ingested by control PMNs was 33.6 ± 4.9. A fraction similar to HE1F-37 but isolated from *E. coli* (which has been reported to be readily killed by bovine neutrophils [3]) was found to have no effect on iodination. PMNs preincubated with HE1F-60 or HE1F-37 at 37°C for 30 min followed by two washes in PBS did not exhibit suppressed iodination when stimulated with opsonized zymosan.

Identification of the components in HE1F-60 and HE1F-37. In addition to having similar biological activity, HE1F-60 and HE1F-37 also shared the following chemical and physical characteristics: maximal UV absorbance at 260 nm, positive reaction of orcinol reagent in the orcinol assay (6), and ability to pass through a membrane with a molecular-weight cutoff of 1,000. Based on these findings, we speculated that HE1F-60 (or HE1F-37) might contain nucleotides or nucleotidelike compounds. To investigate this hypothesis, a solvent system was used which had been previously described for separation of nucleotides or nucleotidelike compounds from bacterial cells on a reverse-phase chromatography column (8). The chromatographic conditions for separating HE1F-60 (or HE1F-37) consisted of solvent A (5 mM tetrabutylammonium phosphate, 4% acetonitrile, 30 mM KH_2PO_4 [pH 6.0]), solvent B (acetonitrile), a 60-min linear gradient (0 to 40% solvent B), and a flow rate of 1.5 ml/min. The peaks present in each sample were identified by the following procedures, which were described previously (5).

(i) Retention time. The retention times of the peaks in each sample were compared to those of reference standards purchased from Sigma. Standards with a retention time similar to that of an unknown peak were coinjected with the

TABLE 1. Effect of "*H. somnus*" fractions on iodination and *S. aureus* ingestion by bovine PMNs

Fraction	% of control (mean ± SE)[a] for:	
	Iodination	*S. aureus* ingestion
HE1F-60	66.3 ± 1.2[b]	102.3 ± 4.1
HE1F-37	54.8 ± 1.6[b]	98.2 ± 4.5

[a] For six PMN preparations.
[b] $P < 0.001$ (level of statistical significance of the difference in iodination values between treated and control PMNs).

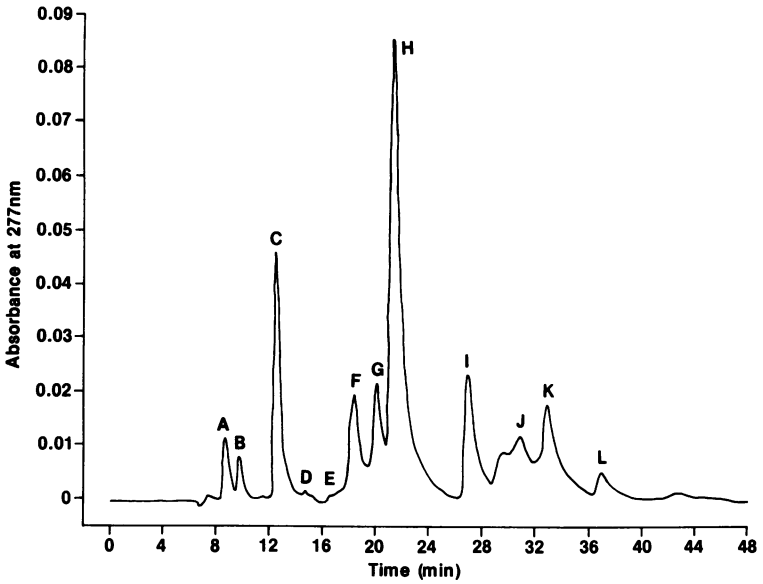

FIG. 1. Reverse-phase HPLC chromatogram of HE1F-60 (100 μl). The chromatographic conditions consisted of solvent A (5 mM tetrabutylammonium phosphate, 4% acetonitrile, 30 mM KH_2PO_4 [pH 6.0]), solvent B (acetonitrile), a 60-min linear gradient (0 to 40% solvent B), and a flow rate of 1.5 ml/min. Peak identities: A, cytosine; B, uracil; C, guanine; D, guanosine; E, adenine; F, CMP; G, UMP; H, GMP; I, AMP; J, CDP; K, GDP; L, ADP.

sample, and changes in peak shape were noted. Standards which caused an unknown peak to increase in height without the appearance of a shoulder or unusual peak broadening were considered to have the same retention time as the unknown peak. Accordingly, the compounds present in HE1F-60 were tentatively identified as cytosine, uracil, guanine, guanosine, adenine, CMP, UMP, GMP, AMP, CDP, GDP, and ADP (Fig. 1). HE1F-37 contained the same components as those identified in HE1F-60 but in different quantities, as judged by the peak heights (Fig. 2). The HE1F-37 fraction isolated from *E. coli* did not contain any of the same components. The presence of cytosine, uracil, guanine, guanosine, and adenine in each "*H. somnus*" fraction was further confirmed by using the same technique but a different set of chromatographic conditions which had been described for the identification of nucleosides and bases (5, 7). The chromatographic conditions consisted of a solvent containing 20 mM KH_2PO_4 (pH 5.7), methanol as a second solvent, 5 min of isocratic elution with the first solvent followed by a 35-min linear gradient to 24% of the second solvent (methanol), and a flow rate of 1.5 ml/min.

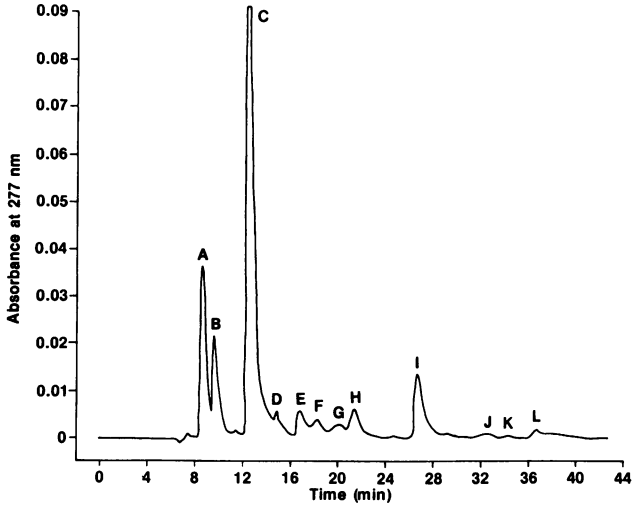

FIG. 2. Reverse-phase HPLC chromatogram of HE1F-37 (100 μl). The chromatographic conditions and the peak identities were the same as those described in the legend to Fig. 1.

(ii) Enzymatic treatments. To further confirm the identities of the ribonucleotides, a sample of HE1F-60 (or HE1F-37) (200 μl) was incubated with 100 μl of 5′-nucleotidase (50 U/ml) at 37°C for 12 h. The reaction mixtures were heated in a boiling water bath for 5 min to deactivate the enzyme before samples were injected into a C_{18} column. HE1F-60 (or HE1F-37) that was incubated with water instead of the enzyme was processed in the same manner and served as a control. For the identification of guanine, 200 μl of the sample was incubated with 100 μl of guanase solution (0.16 U/ml) or 100 μl of H_2O as a control at 37°C for 12 h followed by heating in a boiling water bath for 5 min before a sample of the reaction mixture was injected into a C_{18} column.

When HE1F-60 (or HE1F-37) was treated with 5′-nucleotidase, which catalyzes the cleavage of the phosphate group of nucleotides, a change in the chromatogram was observed. The ribonucleotide peaks either completely disappeared or the peak heights were reduced, while there were new peaks corresponding to uridine, cytidine, and adenosine and an increased peak height for guanosine. A change in the chromatogram also occurred when HE1F-60 (or HE1F-37) was treated with guanase, which deaminates guanine to xanthine. The peak corresponding to guanine disappeared, and a new peak which coeluted with xanthine appeared.

Identification of the suppressive components in HE1F-60 and HE1F-37. Thus far, we had demonstrated that HE1F-60 (or HE1F-37) contained ribonucleotides, a ribonucleoside, and purine and pyrimidine bases which might be involved in the suppression of iodination by bovine PMNs. The next step was to determine which components were involved in the suppression.

When the identified compounds were titrated for their effect on iodination, all of them except cytosine and CDP

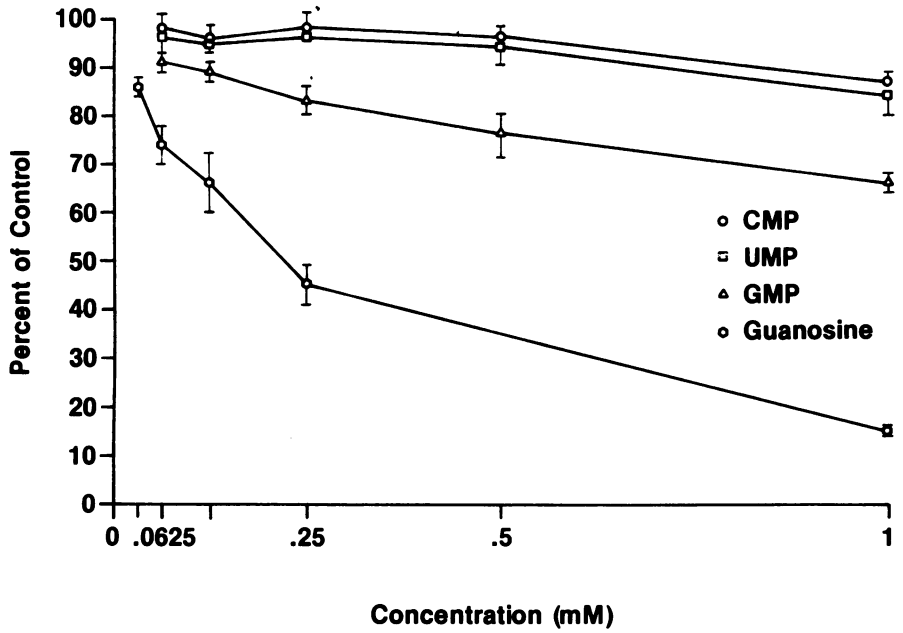

FIG. 3. Effect of various concentrations of CMP, UMP, GMP, and guanosine on iodination by bovine PMNs. Each point represents the mean ± SE for six PMN preparations.

were found to be suppressive, depending on the concentrations used (Fig. 3 through 7). The titration data suggested that guanine, guanosine, adenine, and GMP were the most potent inhibitory compounds. Because the suppression caused by these compounds was dose dependent, the effect of each component on iodination at the concentrations present in HE1F-60 and HE1F-37 could indicate which of them were the major suppressive components. To estimate the concentration of each component present in HE1F-60 and HE1F-37, the peak heights were measured and converted to the corresponding concentrations by using a standard curve generated by injecting known concentrations of the components into the HPLC column and measuring the peak heights. The effect of each component on iodination at the concentrations found in "*H. somnus*" fractions was subsequently determined. The suppression caused by guanine, adenine, and GMP is shown in Table 2. The other components did not cause any significant effect on iodination ($P > 0.05$) at the concentrations found in either fraction, although they were suppressive at higher concentrations. The results, therefore, suggested that guanine and GMP were responsible for most of the suppression of HE1F-60, whereas guanine and adenine were the major suppressive components in HE1F-37. It is noteworthy that guanine, adenine, and GMP at the concentrations found in HE1F-60 or HE1F-37 had no effect on *S. aureus* ingestion and their suppressive effect on iodination could be eliminated by washing the PMNs after exposure to the compounds.

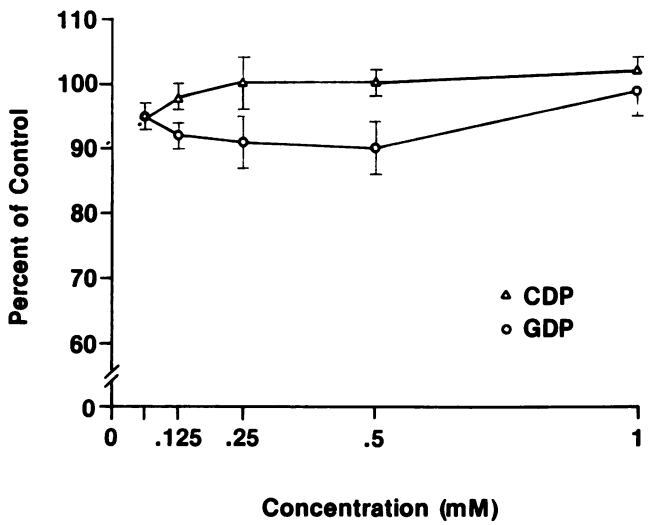

FIG. 4. Effect of various concentrations of CDP and GDP on iodination by bovine PMNs. Each point represents the mean ± SE for six PMN preparations.

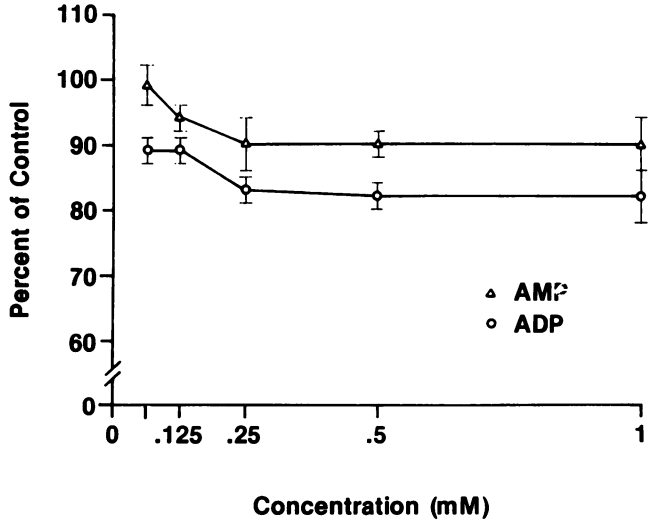

FIG. 5. Effect of various concentrations of AMP and ADP on iodination by bovine PMNs. Each point represents the mean ± SE for six PMN preparations.

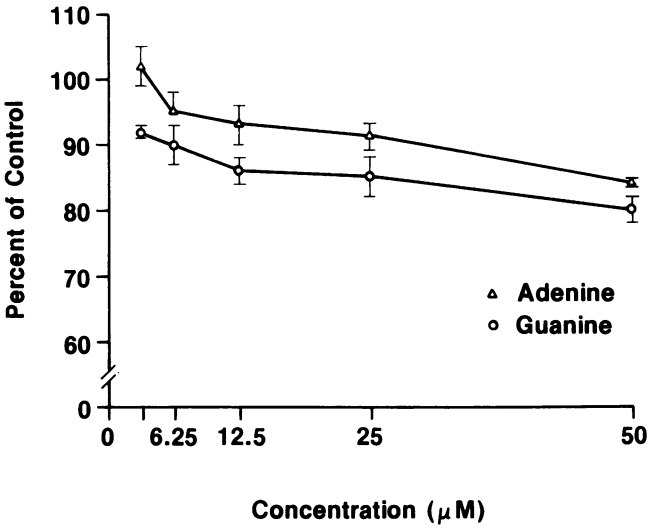

FIG. 6. Effect of various concentrations of adenine and guanine on iodination by bovine PMNs. Each point represents the mean ± SE for six PMN preparations.

TABLE 2. Effect of guanine, adenine, and GMP at the concentrations found in "*H. somnus*" fractions on iodination by bovine PMNs

Fraction	Component (concn [μM][a])	% of control (mean ± SE)[b]
HE1F-60	Guanine (25)	84.9 ± 1.6[c]
	Adenine (3)	100.2 ± 3.5
	GMP (85)	88.8 ± 2.8[d]
HE1F-37	Guanine (70)	74.3 ± 2.4[c]
	Adenine (25)	91.2 ± 1.6[c]
	GMP (10)	100.2 ± 3.2

[a] Final concentration in reaction mixture.
[b] For six PMN preparations.
[c] $P < 0.001$.
[d] $P < 0.01$.

DISCUSSION

In the present study, we identified ribonucleotides, a ribonucleoside, and purine and pyrimidine bases as the components in an "*H. somnus*" fraction which was previously shown to be inhibitory to iodination by bovine PMNs (Hubbard et al., in press). We also demonstrated that "*H. somnus*" could release these compounds into the fluid phase at physiological temperature. Our data suggested that guanine and adenine were the major suppressive components in HE1F-37, whereas guanine and GMP were the primary components involved in the suppression by HE1F-60. Guanine alone accounted for almost half the suppressive activity exerted by either bacterial fraction. However, it was also noted that when the suppressive effects exerted by guanine and adenine (or guanine and GMP) were added together, they did not equal the suppressive effect of HE1F-37 (or HE1F-60). This was probably because of the partial solubility of guanine in aqueous solution with neutral pH. When the stock solution of guanine (10 mM, pH 12.5) was diluted in PBS to the concentrations used (e.g., 0.42 mM, which gave the final concentration of 70 μM in the reaction mixture), some precipitation occurred because of the pH change. Therefore, the actual concentration of pure guanine used in the iodination assay could have been lower than the guanine concentration in the bacterial extracts, in which other components may maintain the solubility of guanine through intermolecular interaction. Nevertheless, we cannot totally exclude the possibility that there is yet another unidentified suppressive component in the "*H. somnus*" fractions.

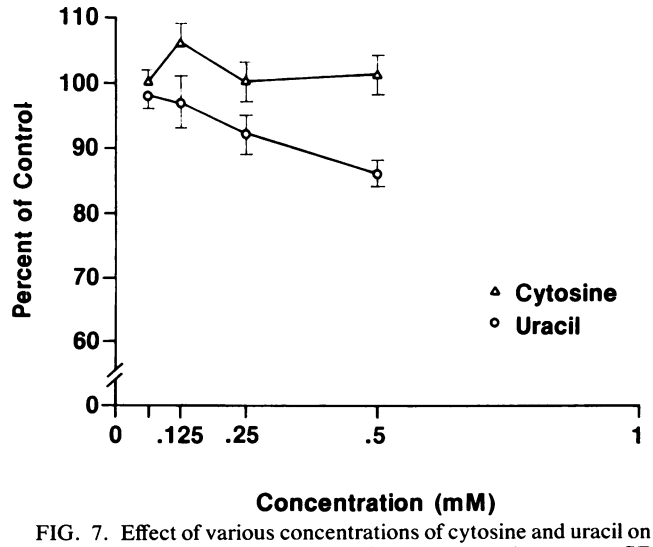

FIG. 7. Effect of various concentrations of cytosine and uracil on iodination by bovine PMNs. Each point represents the mean ± SE for six PMN preparations.

Recently, results of the study by Riches et al. (9) indicated that adenosine, AMP, ADP, ATP, and related structural analogs of adenosine were inhibitory to lysosomal secretion by mouse macrophages. Guanosine was one of the structural analogs that was found to be inhibitory. The functional assay used in that study was different from the one we used in this study; however, both are measures of degranulation by phagocytic cells. Therefore, the inhibition of mouse macrophage lysosomal secretion by AMP, ADP, and guanosine observed by Riches et al. was analogous to our findings for bovine PMNs. Guanine and adenine were not used in their study. An important difference between our observations and theirs is that adenosine, which was the functional molecule in inhibiting lysosomal secretion of mouse macrophages (since AMP, ADP, and ATP had to be converted to adenosine by 5'-nucleotidase to exert the inhibitory effect), did not significantly suppress iodination by bovine PMNs (data not shown). This was probably a result of our use of a different cell type from a different species of animal.

The iodination reaction used here evaluates the activity of the myeloperoxidase-hydrogen peroxide-halide antibacterial system of the neutrophil. The ability of "*H. somnus*" to inhibit neutrophil iodination through the elaboration of purine nucleotides or bases may be important for its intracellular survival. Czuprynski and Hamilton have recently reported that opsonized "*H. somnus*" is readily ingested by bovine neutrophils but is not killed by them (3).

Subcellular fractions isolated from *Brucella abortus* and *Rhodococcus equi* in a similar manner as those from "*H. somnus*" were reported to be inhibitory to iodination by bovine and equine PMNs, respectively (2, 4). Both of these bacteria are also able to survive within phagocytes. The *B. abortus* fraction also contains GMP and adenine as neutrophil-suppressive components (P. C. Canning, J. A. Roth, and B. L. Deyoe, J. Infect. Dis., in press) and was found to preferentially inhibit peroxidase-positive as com-

pared to peroxidase-negative granule degranulation by electron microscopic morphometric analysis (1).

The observations that guanine and adenine were apparently released by "*H. somnus*" at 37°C and that guanine and adenine inhibited the myeloperoxidase-hydrogen peroxide-halide antibacterial activity of the PMN (apparently through inhibition of primary granule degranulation) were both unexpected. It may seem less surprising if one considers that a bacterium destroyed within the phagolysosome would be expected to be degraded by lysosomal hydrolytic enzymes which would liberate nucleotides, nucleosides, and bases from intracellular RNA. These substances may then serve as a signal to stop degranulation into the phagolysosome containing degraded bacteria, thus conserving the remaining lysosomes. A bacterium which has evolved to release purines into its surroundings may be able to inhibit phagosome-lysosome fusion and enhance its intracellular survival by being "perceived" by the phagocyte as already degraded. It would appear to be advantageous to "*H. somnus*" to use purines or purine nucleotides as virulence factors because under normal conditions the host immune system could not be induced to produce humoral or cell-mediated immunity to these compounds because of their small molecular size. The role of purines and purine nucleotides as potential virulence factors of "*H. somnus*" and their mechanism(s) of action require further investigation.

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