In presence of an equimolar concentration of galactose, sorbose could not induce lysis of dog erythrocytes in vitro. Since the catabolism of galactose is independent of hexokinase, and the lactate formation in dog hemolysates was not inhibited at all by sorbose using galactose or glucose-6-phosphate as substrate, the view that sorbose interacts with glycolysis at the level of the hexokinase is strongly supported.

The finding that the sorbose-induced hemolysis in dog erythrocytes is dependent upon time of incubation, temperature, and pH[4] also supports an interaction of sorbose with the red blood cell metabolism rather than a direct action on the cell membrane. In addition, there was no indication of a decreased osmotic resistance of erythrocytes collected from dogs treated with sorbose for 3 weeks[3].

Possible interactions, among others, of sorbose with hexokinase are a) competitive inhibition, b) noncompetitive inhibition and c) inhibition by a metabolite of sorbose. The latter possibility is supported by the time course of inhibition by sorbose of the lactate formation from glucose (table). An increasing inhibition of glycolysis was noted after 6 h of incubation, resulting from a complete inhibition of lactate formation in presence of sorbose between 14.5 and 24 h of incubation, in contrast to the linear increase of lactate formed in absence of sorbose. Enzyme kinetic investigations with partly purified hexokinase suggest that sorbose may be phosphorylated in dog erythrocytes (Keller, unpublished) in contrast to erythrocytes of man[7]. However, sorbose is not metabolized to lactate.

Among several species tested, including man, only dog erythrocytes showed such an extraordinary high sensitivity to sorbose-induced hemolysis[4]. Sorbose did not inhibit the lactate formation in hemolysates from man using glucose, fructose, mannose, and galactose as substrate (Kistler, unpublished). However, in view of the many common forms of hemolytic anemia in humans, and considering the potential role of sorbose as a food additive, further investigations are necessary to exclude the possibility that human erythrocytes with some specific deficiencies could be sensitive to sorbose-induced hemolysis.

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Immunomodulating effects in vitro of a hydroxythiazolobenzimidazole in the absence of mitogenicity

G.H. Warren, L. Mills and H. Friedman

Departments of Microbiology, Wyeth Laboratories, Inc., P.O. Box 8299, Philadelphia, (Pennsylvania 19101, USA), and Albert Einstein Medical Center, Philadelphia, (Pennsylvania 19141, USA), 25 November 1977

Summary. Wy-13, 876, a hydroxythiazolobenzimidazole, enhanced in vitro antibody formation by mouse spleen cells immunized with sheep red blood cells. The optimal dose was 25–50 µg/culture. The compound did not have a mitogenic effect at any dose.

Recent studies suggest that substances such as bacterial lipopolysaccharides stimulate the immune response by inducing mitogenesis[1–3]. Much of the work in this laboratory has been concerned with the role of a synthetic low mol. wt hydroxythiazolobenzimidazole (Wy-13, 876 or 3(p-chlorophenyl)-2,3-dihydro-3-hydroxythiazolo[3,2-a]benzimidazole-2-acetic acid) in antibody synthesis[4]. The compound has previously been shown to inhibit Lewis lung tumor growth and to stimulate T cell activity in vivo[5,6]. The data presented in this study indicate that Wy-13,876 enhances in vitro antibody formation in mouse spleen cells without producing a mitogenic effect.

Materials and methods. Spleen cells obtained from normal 6 to 8 weeks old Balb/c mice were washed in Hank's solution and 5×10^6 cells and cultured in 0.5 ml medium (RPMI 1640) containing 5% fetal calf serum. The cells were placed on a dialysis membrane in the inner chamber of a Marbrook vessel containing 11–12 ml medium in the outer chamber exactly as described elsewhere[7]. The cultures were immunized with 0.1 ml suspensions of sheep red blood cells (SRBCs) ranging from 2×10^4 to 2×10^6 erythrocytes/ml; control cultures contained no SRBCs. The hydroxythiazolobenzimidazole, suspended in tissue culture medium immediately before use, was added at concentrations ranging from 1 to 1000 µg in 0.1 ml medium to test cultures which were then incubated at 37 °C in an atmosphere of 95% air and 5% CO_2 for 5 days. Spleen cells from at least 5 cultures per group were tested for antibody plaque forming cells (PFCs) after incubation by the direct plaque assay of Jerne as described earlier[7].

To determine mitogenic responses, 10^6 spleen cells were incubated with graded quantities (1–500 µg/0.1 ml) of the benzimidazole at 37 °C for 24–48 h and then pulsed with 0.1 ml ^{3}H-thymidine (2 µCi) followed by incubation for an additional 18 h. The amount of radioactivity in the DNA of the cells was determined by standard scintillation counting.

Results. Addition of graded amounts of the benzimidazole to normal spleen cells cultured in vitro induced a change in the antibody response (table 1). Although 1–10 µg of the compound had no significant effect on the number of antibody forming cells in cultures immunized with 2×10^6 SRBCs, a 25–50 µg dose gave an optimal increase of about 50–80%. The 100 µg dose had little effect, while the 500–1000 µg doses greatly reduced antibody formation. This appeared to be due to the toxicity of the drug at the higher levels (table 2). When lower immunizing doses of SRBCs (2×10^5 or 2×10^4) were used, fewer PFCs appeared in the cultures. Addition of graded quantities of the benzimidazole resulted in a greater response at most levels in comparison to control cultures (table 1). Although total antibody formation was less than the level achieved with the highest dose of SRBCs, the percentage increase in PFCs

Table 1. Effect of a hydroxythiazolobenzimidazole (Wy-13,876) on primary in vitro PFC responses by normal mouse spleen cell cultures immunized with SRBC

Culture chamber addition (μg/ml)*	PFCs per 10^6 spleen cells immunized with indicated dose of SRBC** 2×10^6	2×10^5	2×10^4	None
None (control)	971 ± 137	431 ± 64	193 ± 62	23 ± 5
Wy-13,876				
1	833 ± 92	-	-	29 ± 10
10	1062 ± 165	548 ± 72	276 ± 38	38 ± 17
25	1310 ± 140	712 ± 112	458 ± 48	65 ± 29
50	1263 ± 182	810 ± 76	588 ± 100	81 ± 32
100	960 ± 110	531 ± 46	215 ± 43	29 ± 12
500	281 ± 49	235 ± 31	68 ± 22	15 ± 12
1000	48 ± 19	-	-	16 ± 8
E. coli LPS 50	1514 ± 276	812 ± 62	497 ± 59	72 ± 16

* 5×10^6 spleen cells from normal Balb/c mice immunized in vitro with indicated dose of SRBC and treated with graded concentrations of Wy-13,876 or with *E. coli* LPS as a standard. ** Mean PFC response (± SE) 5 days after in vitro immunization of 5-6 cultures with indicated dose of SRBC in at least 2-3 experiments.

with the 10-100 μg doses, in comparison to controls, surpassed the response obtained with 2×10^6 SRBCs. Again the highest doses (500-1000 μg) appeared to suppress the responses because of cytotoxicity.

The addition of benzimidazole also increased 'background' antibody responses in cultures without SRBCs. Such background is always found in the spleen of normal mice prior to immunization and is widely assumed to reflect prior exposure of conventional mice to antigens in the environment cross-reacting with sheep erythrocytes, such as Forssman-antigen positive bacteria in the intestine. The optimum background response occurred in cultures treated with the 25-50 μg dose of benzimidazole. Lower and moderately higher doses gave little stimulation and, similar to the antigen-induced responses, 500 and 1000 μg doses resulted in decreased numbers of PFCs.

Most immunological stimulators are considered to be mitogenic and to enhance immunity by non-specific 'polyclonal' activation of background B lymphocytes. As is evident in table 2, none of the doses of benzimidazole used in this study significantly altered the rate or quantity of thymidine uptake by normal spleen cultures. The 10-100 μg doses, which were immunostimulatory, did not stimulate blastogenesis. The highest doses decreased thymidine uptake, but this again appeared due to cytotoxicity. Thus, the benzimidazole did not appear to be itself a mitogen for murine lymphocytes.

Table 2. Effect of graded amounts of hydroxythiazolobenzimidazole (Wy-13,876) on thymidine uptake by cultures of normal mouse spleen cells

Stimulus in vitro (μg/culture)*	Viable cell number ($\times10^6$)	cpm**	Stimulation index***
None (controls)	1.63	3 551 ± 652	-
Wy-13,876			
1	1.40	6 384 ± 2 050	1.8
10	1.59	4 494 ± 1 750	1.3
50	1.82	4 105 ± 873	1.2
100	1.52	3 929 ± 798	1.1
500	0.95	1 374 ± 650	0.4
PHA (10^{-2})	1.52	21,072 ± 2 500	5.9
LPS (50 μg)	1.40	34,400 ± 3 160	9.7

* 2×10^6 spleen cells from normal Balb/c mice incubated in vitro with indicated dose of Wy-13,876, or, as standards, PHA or LPS, for 72 h and then pulsed for 18 h with ^3H thymidine. ** Mean cpm for 5-6 cultures (± SE) after 10 min count. *** Index = mean cpm test group/mean cpm control.

Discussion. The results of previous in vivo and in vitro studies had indicated the potential anti-tumor activity and immunological potency of Wy-13,876, a hydroxythiazolobenzimidazole[4-6]. Since these results were qualitative, the present study was undertaken to provide information about the action of this compound at the level of single antibody-forming cells. The results obtained indicate that the compound stimulates in a dose dependent manner a moderate but consistent increase in antibody secreting cells in both SRBC treated and untreated cultures. Thymidine uptake was not affected, indicating that this compound, unlike many other immunologic adjuvants, has no mitogenic activity. Bacterial lipopolysaccharides (endotoxin) from gram-negative organisms are considered the classic example of mitogenic adjuvants. Recent studies in this and other laboratories have indicated that detoxified endotoxins and small mol. wt derivatives from such endotoxins, as well as other small mol. wt substances from bacteria, may serve as small mol. wt adjuvants despite the lack of mitogenic activity. The compound examined in this study, i.e., Wy-13,876, thus appears similar to small mol. wt natural products which, despite a lack of mitogenicity for murine lymphocytes, nevertheless can stimulate antibody production as well as background antibody responses. Therefore, it seems likely that mitogenicity is not an absolute requirement for adjuvanticity, despite the probability that at some stage of antibody formation cell division does take place. It seems likely that the antigen, not the adjuvant, may provide the necessary stimulatory signal for immunocytes or their precursors to divide.

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