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Immunosuppression by Hydroxystilbamidine Isethionate, a Lysosome-Stabilizing, Anti-Proteolytic, and Antifungal Drug

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Hydroxystilbamidine (HSB) is a potent suppressor of the plaque-forming cell response of mice injected with heterologous erythrocytes. HSB, given in varying doses and injection schedules, suppressed both the primary and secondary immune responses to bovine serum albumin. Apparently the effect is not simply a toxic effect on spleen cells, because there was no appreciable difference in cell numbers between control and HSB-treated mice. The effect of HSB was most apparent in the early phase of the immune response.

Many drugs have been studied with regard to their ability to modulate the immune response (9). This study describes the immunosuppressant capacity of hydroxystilbamidine isethionate (HSB), a drug reported to have a rather unusual combination of properties. It is antitrypanosomal, antimalarial, antifungal, and carcinostatic (15). Moreover, HSB belongs to the diamidines, a large family of biochemically and pharmacologically interesting compounds. In many situations, especially in the treatment of blastomycosis, large quantities of HSB have been given to patients over long periods of time. Our data, obtained in mice with doses proportional to those used in humans, indicate not only that HSB acts as an immunosuppressant but that it exerts this action at certain critical points in the immunologically relevant tissues and cells.

The exact mechanisms of action of this drug are unknown, but its actions on extranuclear deoxyribonucleic acid (DNA) and lysosomes are the most significant. With *Trypanosoma cruzi* there is selective binding of the drug to the kinetoplastic DNA (4), and in extranuclear DNA in yeast HSB causes petite mutations (6). Both of these reports suggest HSB has an affinity for extranuclear DNA in which it may cause changes. HSB also influences lysosomal structures. Allison and Young (2) presented evidence that HSB is taken up by lysosomes of phagocytes and remains sequestered there. McAdam and Williamson (8) reported that HSB treatment resulted in an increase in the number of lysosome-like bodies and secretion granules in *Trypanosoma rhodesiense*. Geratz (7) reported that HSB and other aromatic diamidines are capable of inhibiting some proteases. HSB (16) was also found to be capable of stabilizing lysosomal membranes. Our report deals with the immunosuppressive effect of HSB.

A lysosomal stabilizer might be expected to suppress or retard the immune response. This prediction follows from the work of Dresser (5) and Spitznagel and Allison (14), who presented evidence that lysosome labilizers, which facilitate release of enzymes from or entry of certain substrates into lysosomes, act as powerful immunological adjuvants. The many known labilizers include vitamin A, endotoxins, streptolysins O and S, and lysolecithin. A lysosomal stabilizer, on the other hand, is defined as an agent which retards release of enzymes from or reduces entry of substrates into lysosomal granules. Though not many lysosomal stabilizers are known, one of them, cortisol, is immunosuppressive. Another, chloroquine, is also immunosuppressive, but less so. To test further the predicted immunosuppressant action of HSB, we studied its effects on the immune response in mice. In our experiments HSB did inhibit spleen cell immune responses to two T cell-dependent antigens as reflected by depressed plaque-forming cell (PFC) responses to sheep erythrocytes (SRBC) and humoral antibody responses to bovine serum albumin (BSA).

MATERIALS AND METHODS

Animals. Adult female CBA mice weighing 20 to 22 g were used throughout these experiments. Mice were obtained from Jackson Laboratories, Bar Harbor, Me.

Reagents. SRBC were obtained from Robbin Laboratories, Chapel Hill, N.C., or Granite Diagnostics, Inc., Burlington, N.C. SRBC were washed three times in phosphate-buffered saline, pH 7.2, and adjusted to

4×10^8 cells/ml; 0.5 ml was injected intraperitoneally. HSB was obtained from Wm. S. Merrell Co., Cincinnati, Ohio. Injections were made intraperitoneally in phosphate-buffered saline, pH 7.2. Tissue culture medium (TC 199) was obtained from Grand Island Biologicals Co., Grand Island, N.Y. BSA Cohn fraction V powder was obtained from Armour Laboratories, Chicago, Ill.

Immunization procedures. (i) Particulate antigens. Mice (six per group) were given various doses of HSB 3, 2, and 1 day before antigen. Other groups were given HSB 1 or 2 days after the injection of antigen. Another group of mice received antigen and HSB simultaneously. A control group received only antigen. The antigen dose consisted of 2×10^8 SRBC. Four days after the injection of SRBC, the mice were sacrificed and spleens were removed and assayed for PFC by the Cunningham (3) modification of the Jerne plaque assay. Assays were carried out in TC 199 and on microscope slides.

(ii) Soluble antigen. Mice (six per group) were given various doses of HSB before, simultaneously with, or after the injection of 1,000 μg of BSA. After 20 days the mice were bled and each was given a booster dose of 300 μg of BSA. After 10 days the mice were bled again. The serum from each mouse was then assayed for antigen-binding capacity by a modification of the Farr technique (14). The $\log_{10}$ geometric mean microgram antigen-binding capacity per milliliter of serum and the $\log_{10}$ standard error of the mean were calculated for each group of six mice.

RESULTS

Suppression of the primary immune response to heterologous erythrocytes. HSB was administered intraperitoneally into mice according to various injection schedules. Each group of mice received a single injection of 500 μg of HSB. HSB was given before, simultaneously with, or after SRBC injection. A control group received only SRBC. HSB was an effective suppressor of the PFC response when given before SRBC stimulation (Fig. 1). If given after injection of antigen or simultaneously with antigen, HSB reduced the PFC response but not to the same degree. In other experiments, HSB and SRBC were administered intravenously rather than intraperitoneally. Identical results were obtained.

Effect of dose of HSB on the primary immune response. We gave the mice single intraperitoneal injections of different doses of HSB 3 days before injection of SRBC. Doses from 50 to 1,000 μg were tested. A dose of 1,000 μg was most effective in suppressing the PFC response of the mice (Fig. 2). This dose resulted in the death of about 10 to 15% of the mice receiving it. Consequently, a dose of 500 μg was chosen for subsequent experiments. This dose gave maximal suppression with minimal toxicity.

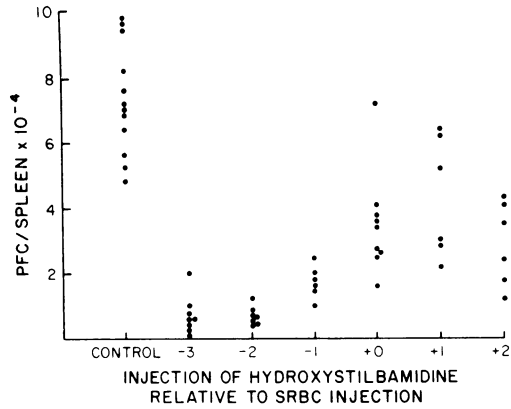

FIG. 1. *Effect of time of HSB injection in relation to injection of SRBC. A single 500-μg injection of HSB was given to groups of mice from 3 days before SRBC (−3) until 2 days after SRBC (+2). The "0" represents HSB and SRBC given simultaneously. The control group received only SRBC. The spleens were removed from all mice 4 days after injection of SRBC. Each dot represents a single mouse.*

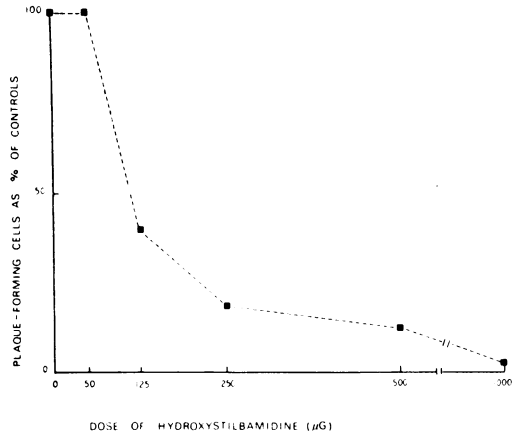

FIG. 2. *Effect of dose of HSB on the primary immune response to SRBC. CBA mice were given a single injection of 125, 250, 500, or 1,000 μg of HSB before injection of SRBC. Four days after SRBC injection, spleens were removed and assayed for PFC. Control group received only SRBC.*

Effect of HSB on spleen cell numbers. To determine if HSB was simply toxic to spleen cells, 15 mice were injected intraperitoneally with 500 μg of HSB. Three days later the mice were sacrificed, spleens were removed, and total nucleated spleen cells were counted in a hemocytometer. Controls consisted of mice which had been given sterile saline intraperitoneally. The numbers of spleen cells recovered from HSB treated mice equaled the numbers from control mice.

Effect of HSB on the appearance of PFC in primary response mice. To see if HSB merely delayed the appearance of PFC, 500 μg was injected into one group of mice and 3 days later SRBC were injected. A control group received only SRBC. Beginning 4 days after antigen and every 2 days thereafter, six mice were removed from each group and assayed for PFC (Table 1). The peak of the PFC response in the controls occurred at day 4. HSB, as anticipated, depressed the plaque response of the treated mice. The peak of their response, diminished as it was, nevertheless occurred at 4 days. Fewer PFC were observed in HSB-treated mice throughout the experiment, but the level of suppression decreased with time. By day 14, the number of PFC observed in both the HSB-treated mice and the control group was essentially at the background level.

Suppression of immune response to BSA. The effect of HSB on the humoral antibody response to a soluble protein antigen was studied using BSA as antigen. This response was quantitated with the serum antigen-binding capacity assay. Table 2 shows the serum antibody-binding capacity of mice injected with BSA with and without HSB. All injections were made intraperitoneally. HSB-treated mice received daily injections of different doses for 3 days preceding injection of BSA. A primary injection of 1,000 μg of BSA was given intraperitoneally 1 h after an intraperitoneal injection of 2 μg of *Escherichia coli* lipopolysaccharide. The control group received 1,000 μg of BSA and lipopolysaccharide. HSB suppressed both the primary response and the secondary response to BSA. However, HSB appeared to be more effective in suppressing the primary response. The lower panel of Table 2 shows a group of mice that were given 500 μg of HSB for 3 days before BSA and daily for 20 days after the injection of BSA. In that case, the suppressive effect was no greater than that observed when HSB was given only before BSA.

TABLE 1. *Effect of HSB on appearance of PFC in mouse spleens*

Days[a]	Controls[b] (PFC/spleen)	HSB[c] (treated mice; PFC/spleen)
4	45,000[d]	8,300
6	14,800	7,100
8	4,750	3,660
10	2,750	1,130
14	900	600

[a] Days after injection of SRBC.
[b] SRBC alone (2×10^8 intraperitoneally).
[c] 500 μg of HSB given intraperitoneally 3 days before SRBC.
[d] Average of at least six mice per group.

TABLE 2. *Serum antibody concentration produced in CBA mice in response to BSA with and without HSB*[a]

HSB (μg injected per dose[a])	Serum ABC ± SE[b]	
	Primary[c] (1,000 μg)	Secondary (300 μg)
None	0.62 ± 0.12	4.76 ± 0.17
None	0.65 ± 0.11	5.70 ± 0.11
500	0.03 ± 0.20	1.83 ± 0.17
250	0.04 ± 0.32	3.28 ± 0.05
125	0.02 ± 0.16	2.88 ± 0.09
500[d]	0.04 ± 0.31	2.72 ± 0.05

[a] All injections were intraperitoneal. HSB was given daily for 3 days preceding injection of BSA and lipopolysaccharide.
[b] The serum antigen-binding capacity (ABC) is the $\log_{10}$ of the geometric mean microgram iodinated BSA/milliliter of serum calculated for a group of six mice. SE, Standard error values in $\log_{10}$.
[c] BSA injected intraperitoneally 1 h after an intraperitoneal injection of 2 μg of *E. coli* lipopolysaccharide. The primary dose was 1,000 μg of BSA followed by a booster dose of 300 μg of BSA 21 days later.
[d] HSB was given daily for 3 days before antigen and then daily for 20 days after injection of antigen.

DISCUSSION

The data presented in this paper indicate that HSB may serve as an immunosuppressant in mice. When given before antigen, HSB is very effective at suppressing the immune response to both particulate and soluble antigens as are several other agents. Alkylating agents, such as L-phenylalanine mustard, fall into this category and have been shown to suppress antibody to SRBC if given before antigen (9). The adrenal steroids as well as several antibiotics, such as mitomycin C and actinomycin D, are all apparently immunosuppressive if given prior to antigenic stimulation. HSB also had some effect if given simultaneously with antigen but less effect if given after antigenic stimulation.

Apparently HSB does not suppress immune responses by massive destruction of lymphocytes. Injection of large doses of HSB did not result in decreases in the numbers of spleen cells recovered, and there may actually have been a slight increase in spleen cells recovered from treated mice as opposed to untreated mice. It seems more likely that HSB treatment resulted in a malfunctioning of some cell involved in the immune response. Since HSB inhibits the immune response if given before antigen, the cell

involved may well be involved in the early processing stages of the immune response.

The dose of HSB required for immunosuppression varies somewhat. In most cases, however, a single dose of 500 μg before antigen was effective in lowering the immune response by greater than 75%. A dose of 1,000 μg usually resulted in greater than 95% reduction of PFC, but was also generally toxic. In some experiments, doses of as little as 50 μg resulted in suppression. Very little is known about elimination of this drug, and it may actually accumulate at some site in the body. Allison and Young reported that HSB remains in lysosomal structures of cells for long periods of time (2). In our experiments with mice, the dose of drug (500 μg) was roughly 10 times the equivalent daily dose of 225 mg received by a person (150 lb) during treatment for blastomycosis. Humans may receive such doses repeatedly for prolonged periods.

The exact mechanism of suppression by HSB is not known. It is possible that the drug is taken up by a cell involved in the processing of antigen, such as the macrophage. HSB remains in the macrophage lysosomal granules where it stabilizes the membranes which then stop functioning. There is evidence for both the uptake of HSB by lysosomes (2) and its stabilizing effects (16). Once the drug is in the lysosomes, the macrophages are essentially blockaded as is possible with the immunosuppressive action of carbon (13) and thorotrast (10). This could explain the effectiveness of the drug when given before or simultaneously with antigen, and fits well the suggestion of Spitznagel and Allison (14) and Munder et al. (11) that adjuvants may act by labilizing lysosomal membranes. In this case, the substance stabilizes lysosomal membranes and is, therefore, immunosuppressive.

It is also possible that HSB may act primarily on lymphocytes rather than on the macrophage. Lysosomal labilizers, such as streptolysin S and staphylococcal alpha toxin, can cause human peripheral lymphocytes, normally inactive in culture, to proliferate. Cortisone, which stabilizes lysosomal membranes, acting as a stabilizer, may inhibit the immune response by inhibiting one of the proliferation steps.

Still another possibility is that HSB may be immunosuppressive because of its ability to inhibit some critical degradative step in the macrophage handling of antigen. For example, HSB has been shown to inhibit certain proteolytic enzymes (7). HSB may inhibit lysosomal proteases and thereby prevent antigen processing by the macrophage. Similarly, gold salts have been shown by Persellen and Ziff (12) to inhibit lysosomal enzymes of guinea pig macrophages, and they may retard inflammatory responses by inhibition of lysosomal enzymes of phagocytic cells.

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LITERATURE CITED

1. Allison, A. C., and L. Mallucci. 1964. Lysosomes in dividing cells, with special reference to lymphocytes. Lancet **287**:1371-1373.
2. Allison, A. C., and M. Young. 1964. Uptake of dyes and drugs by living cells in culture. Life Sci. **3**:1407-1414.
3. Cunningham, A. J., and A. Szenberg. 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology **14**:599-600.
4. Delain, E., C. H. Brack, G. Riow, and B. Festy. 1971. Ultrastructural alterations of *Trypanosoma cruzi* kinetoplast induced by the interaction of a trypanocidal drug (hydroxystilbamidine) with the kinetoplast DNA. J. Ultrastruct. Res. **37**:200-218.
5. Dresser, D. 1970. Adjuvanticity of vitamin A. Nature (London) **217**:527-529.
6. Festy, B., and M. Daune. 1973. Hydroxystilbamidine. A nonintercalating drug as a probe of nucleic acid conformation. Biochemistry **12**:4827-4834.
7. Geratz, J. D. 1969. Inhibitory effect of aromatic diamidines on trypsin and enterokinase. Experientia **25**:1254-1255.
8. McAdam, R. F., and J. Williamson. 1972. Drug effects on the fine structure of *Trypanosoma rhodesiense*: diamidines. Trans. R Soc. Trop. Med. Hyg. **66**:897-904.
9. Makinodan, R., G. W. Santos, and R. P. Quinn. 1970. Immunosuppressive drugs. Pharmacol. Rev. **22**:189-246.
10. Michael, J. G., and C. Murray. 1970. The effect of a blockading agent on the primary immune response. J. Immunol. **105**:411-415.
11. Munder, P. G., R. Gerber, M. Modolell, and H. Fischer. 1968. The influence of various adjuvants on the metabolism of phospholipids in macrophages. Int. Arch. Allergy **36**:117-128.
12. Persellen, R., and M. Ziff. 1966. The effect of gold salts on lysosomal enzymes of the peritoneal macrophage. Arthritis Rheum. **9**:57-65.
13. Sabet, T. Y., and H. Friedman. 1969. Effects of RES blockade on antibody formation. II. Cytokinetics of the secondary hemolysin response and suppressed immunological 'memory' in mice treated with carbon particles. Immunology **17**:535-550.
14. Spitznagel, J. K., and A. C. Allison. 1970. Mode of action of adjuvants; effects of antibody responses to macrophage-associated bovine serum albumin. J. Immunol. **104**:128-129.
15. Weinstein, L. 1970. Antibiotics. IV. Miscellaneous antimicrobial, antifungal and antiviral agents. p. 1304. *In* L. Goodman and A. Gilman (ed.), The pharmacological basis of therapeutics, 4th ed. The MacMillan Co., New York.
16. Weissmann, G., P. Davies, K. Krakauer, and R. Hirschhorn. 1970. Studies on lysosomes. XIII. Effects of stilbamidine and hydroxystilbamidine on *in vitro* and *in vivo* systems. Biochem. Pharmacol. **19**:1251-1261.
17. Weissmann, G., and J. Dingle. 1961. Release of lysosomal protease by ultraviolet radiation and inhibition by hydrocortisone. Exp. Cell Res. **25**:207-210.