5. Kruse, H. D., Schmidt, M. M., MacCollum, E. V., *J. Biol. Chem.*, 1934, v106, 553.

6. Tufts, E. V., Greenberg, D. M., PROC. SOC. EXP. BIOL. AND MED., 1936, v34, 292.

7. Moore, L. A., Sholl, L. B., Hallman, E. T., *Arch. Path.*, 1938, v26, 820.

8. Colby, R. W., Frye, C. M., *Am. J. Physiol.*, 1951, v166, 209.

9. ———, *ibid.*, 1951, v166, 408.

10. Mosher, R. E., *Am. J. Clin. Path.*, 1949, v19, 461.

11. Walser, M., *Anal. Chem.*, 1960, v32, 711.

Received June 6, 1961. P.S.E.B.M., 1961, v107.

Detection of Agents which Interfere with the Immune Response. (26758)

HENRY C. NATHAN, SAMUEL BIEBER, GERTRUDE B. ELION AND GEORGE H. HITCHINGS
Wellcome Research Laboratories, Burroughs Wellcome and Co., Inc. Tuckahoe, N. Y.

Interference with antibody production by cytotoxic drugs, notably antagonists of nucleic acid constituents, has been reported by several investigators(1-8). Substances which exhibit this effect are of interest not only for the insight which they may provide into the immune process, but also for their possible applications to transplantation problems(9,10) and to autoimmune diseases(11).

Screening tests for such materials recently have been devised by Berenbaum(5) and by Sterzl(12) using antigens of bacterial origin. The method to be described herein is based on the agglutinin response in mice to administered sheep cells, and may therefore be regarded as a modification of the procedure used by Makinodan for investigation of radiation effects(13). As will be shown, the modifications which have been made appear to be essential for detection of the effects of drugs. The method as presently employed is simple, economical and capable of providing reproducible results.

Materials and methods. Mice. Specific-Pathogen Free (SPF) Swiss Bagg male mice* weighing 19-21 g were used.

Antigen. Sterile, washed sheep cells† were exposed for 10 minutes at 37°C to an equal volume of 1:10,000 tannic acid in buffered saline solution‡ collected by centrifugation, washed with buffered saline‡(7) and resuspended in the same medium.

Procedure. The initial inoculum of antigen consisted of 0.25 ml of a 30% suspension of cells per mouse, administered intravenously to groups of 10 mice. In the experiments reported herein a second dose of 1.0 ml of a 15% suspension of cells was administered intraperitoneally on the seventh day. The effects of this second dose have been found to be minimal, and in subsequent work it will be omitted. On day 13, the mice were decapitated and the pooled blood was allowed to clot. The serum was used for determination of hemagglutinins.

Hemagglutination. The hemagglutinin titer was determined by serial 2-fold dilution essentially by the method of Stavitsky(14) using 0.1 ml of a 1% suspension of tanned sheep cells per ml of saline-diluted serum. After preparation, the tubes were shaken and then allowed to stand for 18 hours at 5°. Agglutination was then scored 0, 1+ to 4+ described by Stavitsky(14).

Index. The score for each tube was multiplied by the appropriate exponent of the 2-fold dilution series, *i.e.*, a tube showing a 4+ agglutination at a dilution of 2^6 would have a value of 24. These values were then summed for each series. The index of drug effect was obtained as the ratio of these sums for the treated to the untreated control:

$$\text{Antibody Index (A.I.)} = \frac{\Sigma\,(S_1 + 2S_2 + 3S_3 + \ldots . nS_n)\,T}{\Sigma\,(S_1 + 2S_2 + 3S_3 + \ldots . nS_n)\,C}$$

where n refers to the exponent of the dilution

* Darrow Breeding Laboratories.

† Colorado Serum Co.

‡ Saline (0.85% NaCl) is mixed with an equal volume of buffer (29.9 ml M/15 KH_2PO_4 + 76.0 ml M/15 Na_2HPO_4).

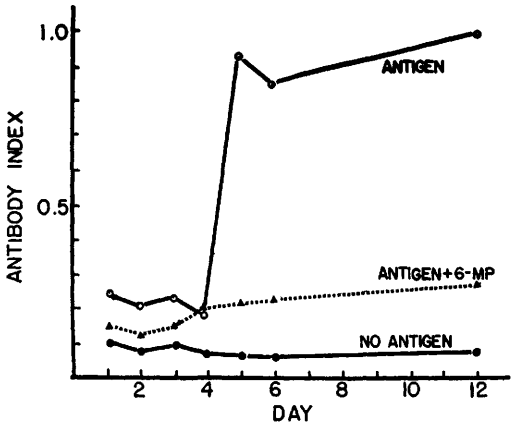

FIG. 1. Appearance of circulating antibody after administration of antigen. ●——● no antigen; ○——○ antigen on day 0; ▲----▲ antigen on day 0, mercaptopurine days 0, 1, 2, 3. Index as described in text.

(=tube number in the 2-fold series), S is the agglutination score for the tube and T and C refer to treated and control series, respectively.

The Σ_c for 28 trials = 175 ± 24.

A value of 0.5 or lower for the index was chosen arbitrarily as the limiting value for activity. However, it will be noted that this is removed from the control average by greater than 3 standard deviations.

Drug therapy. The drugs were given intraperitoneally at the estimated maximum tolerated doses (for initial screening) on 4 successive days, beginning at the time of the first antigenic stimulus. 6-Mercaptopurine (75 mg/kg) was included as a positive control in each experiment, as was a control without antigen or therapy.

Results. The results of hemagglutinin titers determined daily on groups of control mice and mice receiving therapy with 6-mercaptopurine (75 mg/kg/day) are presented in Fig. 1. For the first 4 days the titers were not significantly higher than those of the controls. This is in agreement with the findings of Makinodan(13). The rapid rise in titer on the fifth day is more abrupt than that reported by the previous author, but may be a consequence of the much greater antigenic stimulus provided in the present test. Antibody production is seen to be almost completely suppressed over the period studied when 6-mercaptopurine was administered during the first 4 days.

The relationship between antigenic stimulus, dose of drug and response was explored in a Latin square type of experiment, the results of which are presented in Table I. It will be seen by following each row that suppression of the response increases with increasing dosage of the drug. However, an increase in the antigenic stimulus resulted not in less, but, in fact, a considerably greater suppression. The rather high antigenic stimulus chosen for routine work appears well-adapted to the demonstration of the effects of drugs.

TABLE I. Relationship between Antigenic Stimulus, Dose of Mercaptopurine and Response.

		Dose of mercaptopurine, mg/kg/day × 4			
		0	8.33	25	75
		Index			
Sheep cell suspension*	.11	.78	.74	.50	.46
	.33	.78	.80	.60	.33
	1.0	1.00	.80	.61	.30
	3.0	1.06	—	.04	0

* In proportions of the standard inoculum (1.0) as given in the text.

Time of administration of the drugs in relation to suppression also was investigated. The pertinent data are shown in Table II. Suppression diminishes as treatment is delayed. Even a 24-hour delay appears to have some effect. The effect of single *vs* multiple doses of mercaptopurine also was investigated. In one experiment the usual regime of 4 daily doses at 75 mg/kg gave an index of 0.17, doses of 150 mg/kg on days 0 and 1

TABLE II. Effect of Time of Initiation of Therapy on Suppression of Immune Response.

Time of start	Index
Day 0	.34
1	.46
2	.77
3	1.04
4	.95
Control s therapy	1.00

Each group received only the primary dose of antigen, as described in the text, on day zero. Each of the treated animals was given 4 daily injections of mercaptopurine, 75 mg/kg/day beginning on day indicated.

TABLE III. Effects of Various Agents on Hemagglutinin Response.

Compound	Dose/day, mg/kg	Index
Control without antigen	— (25)	.14 ± .06
Purine analogs		
6-Mercaptopurine	75 (16)	.32 ± .06
6-Thioguanine	2 (4)	.18
Benzimidazole	100	.85
B.W. 57-322*	12.5	.86
	25	.37
	50 (4)	.34
	100	.30
B.W. 57-323*	12.5	.30
6-Methylthiopurine	100 (4)	.42
6-Propylthiopurine	100	.67
8-Azaguanine	50 (2)	.58
Pyrimidine analogs		
5-Bromouracil	500	.69
5-Fluorouracil	25	.84
5-Hydroxyuracil	500	.99
5-Nitrouracil	500	.89
5-Aminouracil	200	.48
2-Thiouracil	50	.61
4-Thiouracil	200	.48
2-Thiothymine	250	.60
4-Thiothymine	200	.67
2-Thiocytosine	100	.63
6-Azauracil	500	.48
6-Azathymine	400	.68
Antifolic acids		
A-methopterin	2 (4)	.49
B.W. 50-276*	7.5 (2)	.51
Miscellaneous		
Nitrogen mustard	10†	.30
	5	.72
Hydrocortisone	37.5	.57
Chlorambucil	7.5 (5)	.47 ± .02
Chloramphenicol	250.0	1.18
Actinomycin C	0.2	.61
Actinomycin D	0.1	.45
Urethane	200	.83

Figures in parentheses refer to No. of trials, where averages of several are presented. Where a substantial No. of trials was involved, stand. dev. has been given.

* B.W. 57-322 6-(1-methyl-4-nitro-5-imidazolyl) thiopurine
B.W. 57-323 2-amino-6-(1-methyl-4-nitro-5-imidazolyl) thiopurine
B.W. 50-276 2,4-diamino-5-(3,4-dichlorophenyl)-6-ethylpyrimidine

† Toxic dose, 5 of 10 mice died.

gave an index of 0.47, and a single dose on day 0 of 300 mg/kg gave an index of 0.51. with 2 of the 10 animals dying of drug toxicity. A number of experiments have indicated that there is no advantage, for the purposes of this test, in extending therapy beyond the 4-day induction period.

Representative screening test results are presented in Table III. It will be observed that 6-mercaptopurine consistently gives a strong repression of the immune response during the period of observation. 6-Thioguanine appears on the basis of a smaller number of trials to be significantly more active than 6-mercaptopurine. The imidazolyl derivatives (B.W. 57-322 and B.W. 57-323) have activities comparable to those of the parent thiopurines. However, B.W. 57-322 appears to be active over a wider range of dosage, and at lower fractions of the maximum tolerated dose which is approximately 150 mg/kg for this dosage schedule. The apparent superiority of this substance in comparison with mercaptopurine has been observed also in maintenance of renal homotransplants in dogs (10). 6-Methylthiopurine but not 6-propylthiopurine appears to have significant activity. 8-Azaguanine, on the basis of 2 trials, would be scored as inactive despite other evidence that in some circumstances it is capable of producing suppression(15). Among the pyrimidine analogs, the apparent inactivities of 5-fluorouracil, the various 2-thiopyrimidines and 6-azathymine are in agreement with the findings of Sterzl(12). On the other hand, 6-azauracil showed borderline activity as did 4-thiouracil, and 5-aminouracil. Two folic acid antagonists, A-methopterin and B.W. 50-276 gave borderline-active results. Nitrogen mustard (HN2) was active only at a toxic dose, but chlorambucil gave very consistent if not very powerful suppression. Actinomycin D was scored as active.

Discussion. The present screening test was designed primarily to find substances which interfere with the inductive phase of the antibody response. For this reason therapy has not been extended to the phase of antibody production. But experiments concerned with the optimal time of therapy show that 6-mercaptopurine, in agreement with the findings of Schwartz(2) has little or no activity once antibody production is established. The selection of the time of the first antigenic stimulus as the optimal time for initiation of therapy is not necessarily at variance with that of Berenbaum(5) who used a different antigen and a single maximal

dose of the test drug 48 hours after administration of antigen.

Where comparisons are possible, the results reported herein are in general agreement with those of Sterzl(12) although some minor differences with regard to the activity of specific compounds will be observed. In the present test to date, the activity with folic acid antagonists has been of moderate dimensions in contrast to the marked effects reported by others(4,12).

Perhaps the most important observation reported here is that the effects of the drug increase with increasing antigenic stimulus. The failure to employ an adequate antigenic stimulus may explain some of the failures to find suppression with 6-mercaptopurine. In the absence of a clear understanding of the processes involved in the inductive phase of antibody formation, the rôle of 6-mercaptopurine remains obscure. However, the present results suggest that maximal drug effects are obtainable only when these processes are stimulated maximally. It is noteworthy that all the substances found active to date also are active antitumor agents in tests with transplantable tumors in mice. The reverse, however, by no means follows.

Summary. A screening test is described for materials which suppress the immune response during its inductive phase. This test is based on the formation of hemagglutinins to sheep red blood cells in mice. 6-Mercaptopurine, 6-thioguanine and their S-imidazolyl derivatives, B.W. 57-322 and B.W. 57-323, show high activity.

The authors wish to acknowledge their indebtedness to Dr. Robert Schwartz for the suggestion which stimulated this investigation, and to thank Dr. R. Y. Calne and Dr. A. W. Frisch for the opportunities to read their papers in advance of publication.

1. Philips, F. S., Hopkins, F. H., Freeman, M. L. H., *J. Immunol.,* 1947, v55, 289.
2. Schwartz, R., Stack, J., Dameshek, W., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 164.
3. Schwartz, R., Eisner, A., Dameshek, W., *J. Clin. Invest.,* 1959, v38, 1394.
4. Uphoff, D. E., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 651.
5. Berenbaum, M. C., *Nature,* 1960, v185, 167.
6. Sterzl, J., *ibid.,* 1960, v185, 256.
7. Condie, R. M., Mennis, W. I., Miller, C., *Fed. Proc.,* 1961, v20, 26.
8. Frisch, A. W., Davies, G. H., *J. Immunol.,* 1961, in press.
9. Calne, R. Y., *Lancet,* 1960, v1, 417.
10. ———, *Transplantation Bull.,* 1961, in press.
11. Dameshek, W., Schwartz, R., *Trans. Assn. Am. Physicians,* 1960, v73, 113.
12. Sterzl, J., *Nature,* 1961, v189, 1022.
13. Makinodan, T., Friedberg, B. H., Tolbert, M. G., Gengozian, N., *J. Immunol.,* 1959, v83, 184.
14. Stavitsky, A. B., *ibid.,* 1954, v72, 360.
15. Dutton, R. W., Dutton, A. H., Vaughan, J. H., *Fed. Proc.,* 1959, v18, 219.

Received June 6, 1961. P.S.E.B.M., 1961, v107.

Effect of Stress on Response to Fludrocortisone Excess in the Rat.* (26759)

C. E. HALL AND O. HALL
Carter Physiology Laboratory, University of Texas Medical Branch, Galveston, Texas

We have reported that in rats treated with desoxycorticosterone acetate (DCA)(1,2) or STH(3), stress increases the hypertensive and/or nephrosclerotic action of the hormone, an effect not noted with cortisol(4). Selye(7) has reported that immature unilaterally nephrectomized rats given 200 μg/day of 9α fluoro-hydrocortisol (fludrocortisone) and 1% NaCl solution to drink, develop severe nephrosclerosis and ascites, but not cardiac lesions, encephalopathy or visceral periarteritis. Blood pressure showed an elevation to hypertensive levels in many animals, but this was not sustained, declining premortally.

It seemed of interest to determine the influence of simultaneous exposure to stress

* This study was supported by a grant from USPHS.