A Rapid Method for *in Vitro* Screening of Immunosuppressants Using Mouse Spleen Cells[1] (35009)

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Inhibition of antibody production by immunosuppressants has been investigated using numerous approaches (1–3). The need for a rapid, but quantitative approach for immunosuppressive activity has been recognized. The advantages of an *in vitro* approach include a more rapid standardization of test conditions for comparing drugs as well as a means of studying the molecular action of immunosuppressants at the cellular level. However, few *in vitro* approaches for rapidly screening immunosuppressants have been described.

Immunization of mice with sheep erythrocytes leads to the rapid production of hemolysin by spleen cells (4, 5). The ability of such cells to produce hemolysin *in vitro* suggested the possibility of using this system to measure the effect of immmunosuppressants on antibody production. The purpose of this paper is to describe such a system and its ability to screen chemicals for immunosuppressive activity.

Materials and Methods. Animals and immunization schedule. Adult Swiss Webster mice, weighing between 25 and 30 g, were injected intraperitoneally with a single 0.5-ml dose of a 5% sheep erythrocyte suspension. The animals were sacrificed 4 days after immunization by cardial bleeding and their spleens were removed aseptically.

Preparation and culturing of spleen cells. After cutting the tip of the spleens, the cells were expressed with the aid of a rubber policeman into phenol red-free Eagles basal medium with Earle's balanced salt solution (EBM). The expressed cells were collected and washed three times with EBM at 4°. After the last wash the spleen cell suspensions were centrifuged at 20*g* for 3 min to separate nucleated cells from red blood cells and debris. The nucleated cells forming the top layer were removed and counted in 2% acetic acid with a hemocytometer. These cells were suspended in EBM plus 3% gamma globulin-free calf serum (Grand Island Biological Co.), supplemented with 100 units of penicillin and 100 μg of streptomycin/ml. Four-ml volumes of this cell suspension were placed in 60-mm petri dishes and incubated in an atmosphere of 95% air and 5% CO_2 at 37°. After appropriate periods of incubation, cells and culture fluids were removed from the plates, separated by centrifugation (500*g* for 10 min) and the supernatant culture fluid was assayed for antibody.

Antibody assay. A standardized suspension of sheep erythrocytes was prepared so that an OD of 0.7 at 541 mμ was obtained when 0.25 ml of the suspension was completely lysed in 3.5 ml of 0.1% $NaCO_3$. To 1 ml of the culture fluid to be assayed was added 0.25 ml of the standardized erythrocytes suspension and the mixture was incubated at 37° for 30 min with occasional manual shaking. After the sensitization period, 0.25 ml of a 1:20 dilution of fresh frozen guinea pig complement (complement titer, 1:50) and 2.25 ml of isotonic Veronal buffer at pH 7.3 (6) was added to all samples except the control. The hemolysin–erythrocyte control received 2.5 ml of isotonic Veronal buffer. The mixtures were incubated for 1 hr at 37° with occasional agitation. After 1 hr, the unlysed cells were removed by centrifugation (500*g* for 10 min) at 4°. The optical density (OD) resulting from the released hemoglobu-

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lin was measured at 541 mμ on Spectronic 20 (Bausch and Lomb). All sample OD readings were corrected for any nonspecific lysis of hemolysin–erythrocyte control.

Immunosuppressant assay. Sterile solutions of each chemical were prepared at 10 times the final test concentration. Spleen cell suspensions were prepared and incubated as previously described. Three hr after plating, each chemical was added to triplicate sets of cultures designated as test while cultures designated as control received no chemical additions. After 24 hr culture fluids were collected individually and assayed for hemolysin. To insure establishment of the cells before testing, an incubation period of 3 hr before the addition of the chemical was necessary.

Results. The ability of the hemolysin assay to measure quantitatively hemolysin present in the culture fluid was tested. Spleen cell suspensions (1.8 to 2.0 $\times$ 10^8 cells) were prepared from immunized mice and cultured for 24 hr. The culture fluids were pooled and diluted for hemolysin assay. The results of a typical experiment in which the quantity of hemolysin was varied and the concentration of complement was held constant (at 1:20 dilution) is shown in Fig. 1. A linear relationship between hemolysin concentration and lytic activity as measured by the absorbance of released hemoglobulin is shown. It is apparent that quantity of hemolysin in the culture fluid can be measured by the portion of the curve that obeys Beer's law. The quantity of hemolysin, therefore, in all subsequent experiments was expressed as hemolytic units per milliliter by assigning a value of one to that amount of hemolysis which gave a OD reading of 0.4 at 541 mμ.

To determine the cell concentration yielding optimal hemolysin titers, spleen cells from immunized mice were plated at different concentrations and cultured for 24 hr. A spleen cell concentration of 1.4 $\times$ 10^8/plate from immunized mice yielded optimal hemolysin titers whereas similar numbers from nonimmunized mice failed to give measurable amounts of hemolysin. At this concentration

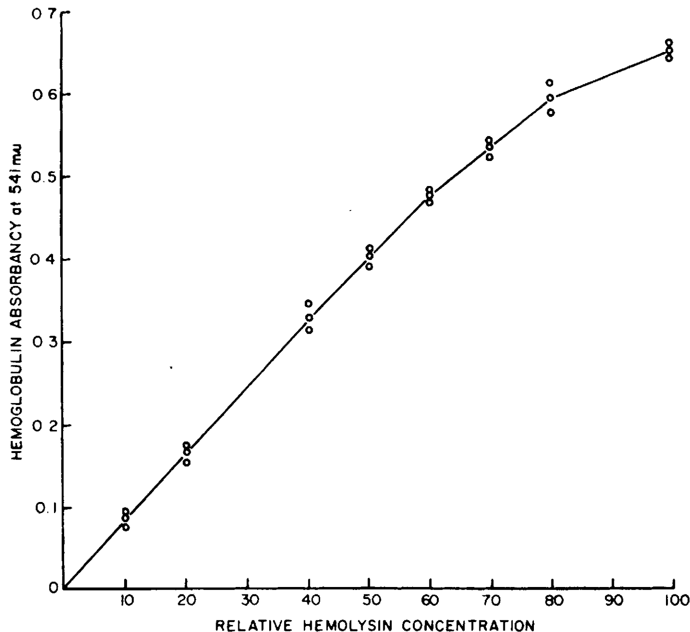

FIG. 1. The relationship between hemolysin concentration and lytic activity at constant-complement dilution. Spleen cell suspensions (1.8 to 2.0 $\times$ 10^8 cells/plate) were prepared from mice 4 days (after) immunization with sheep erythrocytes. After 24-hr incubation, the culture fluids were pooled and diluted for hemolysin assay using 1:20 dilution of complement. The relative hemolysin concentration in the culture fluid was plotted against the resulting absorbance of released hemoglobulin at 541 mμ. Each symbol represents a single hemolysin determination.

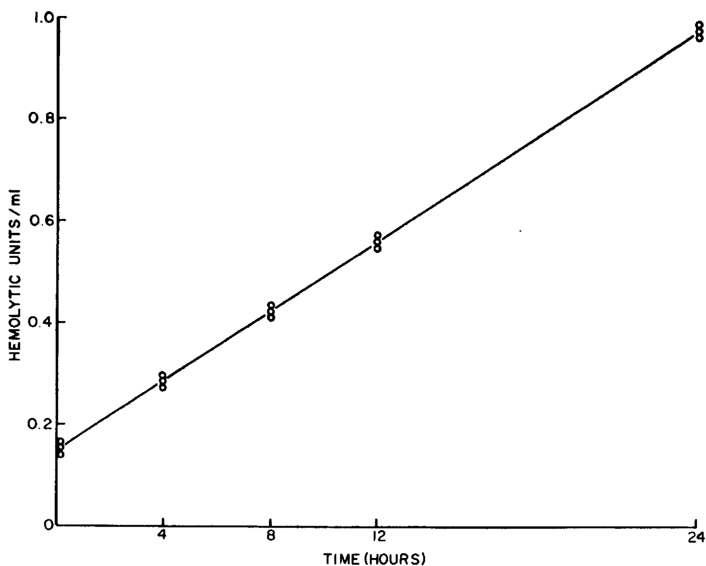

FIG. 2. Kinetics of hemolysin production by spleen cell cultures. Spleen cell cultures (1.4×10^8 cells/plate) were prepared from mice, 4 days after immunization with sheep erythrocytes. Culture fluids were collected at various times during the incubation period and assayed for hemolysin. Each symbol represents the hemolysin titer for one plate.

spleen cell viability measurements by trypan blue exclusion counts showed an average 90% survival after 24-hr incubation. Thus in all subsequent experiments, a spleen cell concentration of 1.4×10^8/plate was used.

The rate of the appearance of hemolysin in the culture fluid was measured over the 24-hr incubation period. Data in Fig. 2 show a linear increase in the amount of hemolysin released into the culture fluid by the spleen

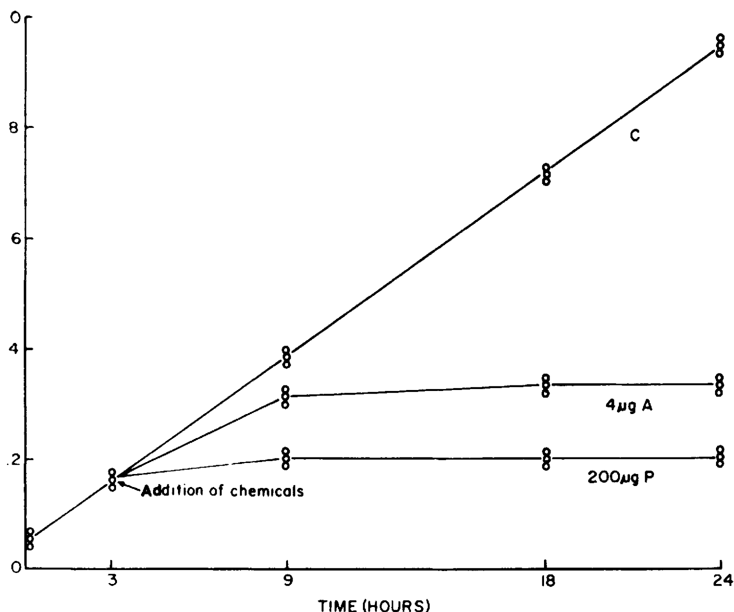

FIG. 3. Effect of actinomycin D (A) and puromycin (P) on the kinetics of hemolysin production (conditions identical to those described in legend to Fig. 2).

cells with respect to incubation time. To ascertain if the increase in hemolysin represented new antibody synthesis *in vitro*, the effects of actinomycin D (1 μg/ml and puromycin (50 μg/ml) in the system was determined. Actinomycin D is a known inhibitor of DNA dependent RNA synthesis (7) whereas puromycin has been shown to inhibit new protein synthesis (8). Puromycin and actinomycin D were added to separate spleen cell cultures 3 hr after plating and hemolysin levels were determined over 24 hr. The results presented in Fig. 3 show puromycin inhibited the increase of hemolysin appearing in the culture fluid immediately or shortly after its addition. The addition of actinomycin D, however, did not immediately inhibit the appearance of hemolysin in the culture fluids. Hemolysin inhibition appeared to occur within 6 hr after the addition of actinomycin D. These results indicate that an increase in hemolysin levels in the culture fluid required new protein synthesis and was not due to the release of previously synthesized protein.

Having demonstrated the appearance of new hemolysin at a linear rate, the effects of chemicals were tested. The results of testing 6-mercaptopurine (6-MP), 6-azauridine (6-AU), 6-chloropurine (6-CP), 6-azathymidine (6-AT), 5-nitrouracil (5-NU), caffeine, and aspidospermine are presented in Table I.

TABLE I. Effect of Chemicals on *in Vitro* hemolysin Production.

Chemical	Conc/1.4 × 10^8 cells (μg)	Inhibition of hemolysin production (%)
6-Mercaptopurine	320	37.3
6-Azauridine	320	35.5
6-Chloropurine	320	21.9
6-Azathymidine	320	0.0
5-Nitrouracil	320	4.0
Caffeine	4000	33.0
Aspidospermine	4000	26.0

[a] Spleen cell suspensions were prepared according to the conditions described in legend of Figure 2. The results represent the mean of triplicate determination.

At the concentrations tested 6-MP, 6-AU, 6-CP, caffeine, and aspidospermine partially suppressed antibody production (22 to 37%) whereas 6-AT and 5-NU showed little or no inhibition (4% or less). Of the chemicals tested only aspidospermine caused a significant reduction of cell viability as measured by trypan blue exclusion suggesting cytotoxic action. The possibility that the chemicals themselves interfered with the hemolytic assay was controlled by the addition of the chemicals separately to culture fluid containing a known hemolytic titer. Reduction of the control hemolytic titer would indicate interference and invalidate the results of suppression *in vitro*. No interference was noted with the chemicals tested at concentrations which inhibited antibody production.

Discussion. The results of the above study show the ability of the assay to measure quantitatively hemolysin produced by spleen cells *in vitro*. The cell cultures were prepared from spleens of mice which had been previously immunized with SRBC. The production of hemolysin was shown to be *de novo* and the rate of appearance was linear with respect to culture time. In addition, chemicals known to have immunosuppressive activity such as 6-mercaptopurine, 6-azauridine, and 6-chloropurine proved to be effective in inhibiting antibody production. Whereas 6-azathymine and 6-nitrouracil, chemicals usually not associated with immunosuppressive activity were found not to give significant suppression. These findings suggest an *in vitro* model for screening chemicals for immunosuppressive activity.

Recently, the plaque forming cell (PFC) assay for measuring antibody producing cells was suggested as a rapid means for screening agents for immunosuppressive activity both *in vivo* and *in vitro* (9). Promising results were obtained when the test agents were administrated *in vivo* and the PFC response was measured 4 days after immunization with sheep erythrocytes. A 50 to 90% reduction of the expected PFC response was found when effective immunosuppressants were tested. However, difficulties were encountered when attempts were made to use the PFC assay for testing immunosuppressants *in vitro*. A 10 to

30% decrease in the number of PFC in untreated-control cultures was found during the 4- to 5-hr incubation period required for testing. The investigators suggested that this difficulty might be overcome by improving the tissue culture conditions.

The advantages of an *in vitro* approach for testing immunosuppressants are preserved by the procedure proposed in this study. In addition the simplicity and economy of this procedure suggest its possible use in programs designed for large scale screening of immunosuppressants and in studies on the mechanism of such drugs. Furthermore, this *in vitro* technique could be incorporated in the National Cancer Chemotherapeutic screening program.

Summary. An *in vitro* system for quantitatively measuring hemolysin production by mouse spleen cells is described. The synthesis of hemolysin was shown to be *de novo* and the rate of appearance was linear with respect to culture time. Known immunosuppressive chemicals were tested using this system and were found to be effective at inhibiting antibody production whereas nonimmunosuppressive drugs were noneffective. These findings suggested that this system would be suitable for rapidly screening chemicals for immunosuppressive activity and may be of aid in determining their mode of action at a cellular level.

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