Studies with the Murine Leukemogenic Rauscher Virus.
III. An in Vivo Assay for Anti-Viral Agents*

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SUMMARY

An in vivo assay system is described for the chemotherapeutic testing of drugs against the leukemogenic Rauscher virus. A dose-response relationship between the degree of splenomegaly and the dose of virus was established in order to employ spleen weight as the parameter for quantitatively measuring virus titer. The parameters of drug testing were retardation of splenomegaly and inhibition of virus synthesis in the drug-treated host animal. The potential anti-viral activity of various drugs tested in viremic animals was determined by sub-inoculating plasma harvested from treated animals into normal recipients.

Six clinically active drugs were tested for anti-viral activity. Of these, 6-mercaptopurine, methotrexate, cyclophosphamide (Cytoxan), and vincristine showed effective anti-viral activity. Of the additional drugs tested, actinomycin D, puromycin, 5-fluorouracil, 6-thioguanine, and 1,3-bis(2-chloroethyl)-1-nitrosourea were also effective. Conversely, prednisone and methylglyoxalbisguanylylhydrazone effected an apparent increase in the yield of virus recoverable from treated animals.

In recent years many investigators have studied the chemotherapy of viral diseases. Numerous efforts have been made to obtain direct anti-viral activity, with a large number of compounds against viruses grown in tissue culture or chick embryo or with diseases produced experimentally in laboratory animals.

Aromatic diamidines (4) and several 1,4-dioxyquinoxalines (21) were effective against the viruses of the lymphogranuloma and psittacosis group. Vaccinia virus was sensitive to numerous enzymatic inhibitors, such as cyanide, atabrine, proflavin, iodoacetic acid, and some amino acid analogs (38). Mice given inoculations intracerebrally of the neurotropic strain of vaccinia virus were protected following treatment with some aromatic or heterocyclic thiosemicarbazones. The protective effect appeared to be anti-viral (27, 39, 40). Of several folic acid antagonists and amino acid analogs tested, aminopterin was effective against vaccinia virus in tissue culture (9), and p-fluorophenylalanine was inhibitory for poliovirus (26) and fowl plague virus (42). Administration of β-phenylserine to rats delayed death from lethal amounts of rabies virus (31).

Antibiotics were also shown to be active anti-viral agents against certain viruses. Chlorotetracycline was effective against the psittacosis virus in vivo (30). Puromycin prevented both maturation and RNA synthesis of poliovirus (26). Heline and Statolon protected mice against ECHO-9 virus (10). Of several enzymes tested for anti-virus activity, phospholipase A inactivated Rous virus in vitro (14), ribonuclease inhibited vaccinia virus in vitro (37), and exposure of three strains of herpes simplex to trypsin rapidly destroyed infectivity (15).

Several oncogenic viruses and their induced tumors have been employed in test systems to evaluate the anti-viral and anti-tumor capacities of various drugs. Groupé et al. (17, 18), Johnson et al. (22, 23), and Bather (1) reported on in vivo studies with the Rous sarcoma virus. Sugiura (35, 36) and Mirand et al. (28) have studied the effect of a variety of compounds on the Friend virus-induced leukemia. Chirigos et al. described in vivo assay systems for the chemotherapeutic testing of drugs against the Moloney virus-induced leukemia (6) and the Rauscher virus-induced leukemia (7, 8).

Despite the availability of several oncogenic viruses, only a few reports have appeared in the literature concerning anti-viral test systems employing oncogenic viruses. Mirand (28), in studies with the Friend virus, demonstrated in vivo and in vitro anti-viral activity of AB-103, AB-132, and 6-mercaptopurine. Groupé et al. (17, 18), using Rous sarcoma virus in vivo, reported on the anti-tumor activity of Xerosin. Johnson (22, 23) described the prophylactic effect of selected compounds...
which extended the latent period to tumor induction by standardized doses of Rous sarcoma virus. The alkylating agents, melphalan and triethylene melamine, appeared to exert anti-viral activity against the leukomogenic Rauscher virus in vitro (7). Huebner reported an inhibition of the oncogenic effects of human adenovirus type 12 in hamsters by treating with 5-iododeoxyuridine (20).

Adult BALB/c mice infected with Rauscher virus develop a potent viremia beginning 3 days after infection and an increase in spleen weight within 7 days post-inoculation. Histologically, the disease is characterized by intense erythropoiesis and generalized lymphoid leukemia (6, 32, 33). The purpose of this study was to develop an in vivo assay system for testing potential anti-viral agents employing spleen weight as a readily measurable parameter of anti-viral activity.

Six clinically active drugs—6-mercaptopurine, methotrexate, cyclophosphamide, prednisone, vincristine, and methylglyoxalbisguanylylhydrazone—were selected for study. Additional drugs reported by others to show antiviral activity were also included in the present investigation.

**MATERIALS AND METHODS**

*Preparation of standard virus.*—The preparation of standard lots of virus has been described previously (7, 32).

*Virus inoculum.*—Preparation of the virus inoculum was carried out as follows: For each experiment a frozen aliquot of a 10 per cent extract of leukemic spleens (P382) was allowed to thaw, and 0.2 ml. was inoculated intraperitoneally into 6- to 8-week-old BALB/c male mice weighing between 21 and 25 grams.

*Randomization of test animals.*—In all experiments mice were randomized shortly after inoculation and then distributed into appropriate groups.

*Drugs* and treatment.—Actinomycin D, puromycin, cyclophosphamide (Cytoxan), 5-fluorouracil (5-FU), 6-thioguanine, vincristine, 1,3-bis(2-chloroethyl)-1-nitrosourea (NSC-409,962), and methylglyoxalbisguanylylhydrazone were dissolved in 0.9 per cent NaCl. 6-Mercaptopurine (6-MP) was dissolved in dilute alkali. Methotrexate (MTX) was dissolved in 2 per cent NaHCO3. Prednisone and p-isopropyl benzaldehyde thiosemicarbazone (NSC-9,936) were suspended in 0.1 per cent methyl cellulose. The drugs were administered subcutaneously in the axillary region in a constant volume of 0.01 ml/gm of body weight. Treatment was initiated 5 days after virus inoculation and administered daily for 4 days (day 5 to day 8). The selection of the dose of each drug was based on results of previous experiments in which the drug level was determined to be at, or near, the maximum tolerated dose for this strain of mouse.

**Scheme for in vivo anti-viral assay.**—The scheme employed for studying the influence of drug on the virus and virus-host relationship is as follows: mice given inoculations intraperitoneally of virus were divided into control and drug-treated groups and served as donor mice for bioassay. Therapy was initiated on the 5th day after infection, at which time the mice were known to be viremic, and continued once daily for a total of four treatments. On the 9th day individual virus-inoculated control and drug-treated donor mice were anesthetized with ether and bled by way of the brachial artery. Blood was collected with a heparinized syringe, and the plasma was separated by centrifugation (2150 X g) for 5 minutes. Spleen weights of these mice were recorded. Serial tenfold dilutions of plasma were prepared in sterile 0.9 per cent NaCl, and 0.2 ml. was inoculated intraperitoneally into groups of recipient mice. At 21 days post-inoculation of donor plasma the recipient mice were killed by cervical fracture, and their spleens were weighed individually on a Torsion balance style DL-T-2. Spleen weights were read to the nearest 10 mg.

**RESULTS**

*Response of splenomegaly to dose of virus.*—This study was designed to show that spleen weight at various intervals after inoculation was a reliable indication of the amount of virus contained in the infecting dose. The data presented in Chart 1 illustrate the results of this study. Groups of mice given inoculations of various dilutions of virus were killed at intervals after inoculation, and their spleen weights were determined. The mean log spleen weights of five mice are plotted against the infecting dose of virus. The data show that the rate and degree of splenomegaly are dependent on dose of virus and may be used to measure the amount of virus contained in preparations of unknown potency.

*Experimental.*—With the use of the in vivo anti-viral system, twelve drugs were tested for anti-viral activity. The results are presented in Charts 2A and B, and 3A and B, where mean log spleen weight is plotted for each group of donor and recipient mice. Table 1 gives the results of fitting a straight line to the data for each recipient group. Results shown in Chart 1, Chart 2A and B, and Chart 3A and B indicate that log spleen weight is linearly related to log virus dilution. For each group the fitted line has the form y = a + b (x - x) where y is log spleen weight, x is log dilution, and x is mean log dilution. The slope b is the average of the five slopes obtained by fitting (by the method of least squares) separate lines from recipients sharing a common donor. The mean log spleen weight is a.

The fitted straight lines (Charts 2A and B and 3A and B), which represent the response in recipient animals

1 Obtained from the N.I.H. breeding colony.
2 Vincristine sulfate was obtained from Eli Lilly and Co.
3 The Torsion Balance Co., Clifton, N. J.
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Chart 1.—Relation of mean log spleen weight (mg.) response to dose of virus at five intervals after virus inoculation.

Chart 2A and B.—Mean log spleen weights (mg.) for control and drug-treated donor mice and recipient mice given inoculations of serial dilutions of donor plasma. Most means based on five animals. Only undiluted plasma from 5-day control donors was inoculated into recipients in this experiment (2A).
given inoculations of donor plasma, facilitate over-all comparison of the effect of various drugs on viremia in treated and untreated donor animals. The comparisons can be made by comparing the rate of change of spleen weight with donor plasma dilution (a difference in slope $b$) or in mean log spleen weight (a difference in $a$). The slopes ($b$) and mean log spleen weight (a) and their associated standard deviations are summarized in Table 1.

The degree of viremia in donor animals on the day therapy was initiated (day 5) is reflected by the spleen weights of recipient animals given inoculations of serial dilutions of control donor plasma (Control day 5, Charts 2A and B and 3A and B). As anticipated (see Chart 1) the data show that a direct relationship exists between spleen weight and dose of virus. The data also show that donor animals sacrificed on day 5 were viremic, although their spleens had not markedly increased in weight.

As shown in Chart 2A, three drugs retarded splenomegaly in the treated donors, with actinomycin D producing the more extensive therapeutic effect. The mean log spleen weights (a) of recipient animals given inoculations of serial dilutions of control viremic donor plasma (Table 1, Group 2), were compared with those of recipient animals given inoculations of drug-treated donor plasma (Table 1, Groups 3–5). The significantly lower amount of recoverable virus observed (Groups 3 and 4) suggests that plasma of donor animals treated with actinomycin D and puromycin contained less virus and is indicative, therefore, of an $in vivo$ anti-viral effect. $p$-Isopropyl benzaldehyde thiosemicarbazone (NSC-9,936) did not exert any significant anti-viral activity (Group 5).

6-MP, MTX, and cyclophosphamide were effective in retarding splenomegaly in the donor animals (Chart 2B) and also exerted an extensive anti-viral effect. A comparison of mean log spleen weights of recipient animals given inoculations of serial dilutions of control viremic donor plasma (Table 1, Group 2) with those of recipients given inoculations of drug-treated donor plasma (Table 1, Groups 3–5) indicated that the virus titer in the drug-treated donor animals at the time of sacrifice was decreased by at least 2 logs of virus.

Although spleen weights in mice treated with prednisone were lower than those in untreated control animals, prednisone was shown not to have anti-viral activity following bioassay of donor plasma in recipient mice (Chart 3A). Indeed, the higher mean log spleen weights of recipient animals receiving prednisone-treated donor plasma (Table 1, Group 4), as compared with those of the controls (Table 1, Group 2), suggest an increase in viral yield of 0.2 logs. The results obtained with prednisone illustrate the value of employing recipient bioassay animals to distinguish between a nonspecific spleen-retarding effect and true anti-viral activity in the treated animal. The data in Chart 3A also show that 6-thioguanine and 5-FU were effective in retarding spleen weights of donor animals. The lower mean log spleen weights of recipient mice given inoculations of the treated donor plasma (Table 1, Groups 3 and 5), when compared with those of controls (Table 1,
TABLE 1
RESULTS OF FITTING STRAIGHT LINES TO THE DATA FOR EACH RECIPIENT GROUP

<table>
<thead>
<tr>
<th>Chart</th>
<th>Donor Mice</th>
<th>Recipient mice*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group no.1</td>
<td>Drug</td>
<td>Dose (mg/kg)</td>
</tr>
<tr>
<td>3A</td>
<td>1 None</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2 None</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>3 Actinomycin D</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>4 Puromycin</td>
<td>70</td>
</tr>
<tr>
<td>3B</td>
<td>1 None</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2 None</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>4 MTX</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5 Cytoxan</td>
<td>70</td>
</tr>
<tr>
<td>4A</td>
<td>1 None</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2 None</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>3 5-FU</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>5 Prednisone</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>5-Fluorouracil</td>
<td>5</td>
</tr>
<tr>
<td>4B</td>
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<td></td>
<td>2 None</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>3 Vincristine</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>4 NSC-409962</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>5 Methyl-GAG</td>
<td>50</td>
</tr>
</tbody>
</table>

* Results of donor plasma bioassay in recipient mice. Recipient animals sacrificed 21 days post-inoculation with donor plasma.
† Mice given inoculations intraperitoneally of 0.2 ml. of 10^-2 virus dilution (P-382).
‡ Treatment was initiated 5 days after virus inoculation and administered daily for 4 days (days 5 through 8).
§ Δa and Δb based on nine observations for 5-day control groups and fifteen observations for all other groups.
# sΔa = standard deviation of Δa; sΔb = standard deviation of Δb.
¶ The difference between treated and control donor animals in the amount of recoverable virus (log) following assay of donor plasma in recipient mice. The amount of recoverable virus was estimated by comparing the mean log spleen weight (a) to the 3-week standard curve, Chart 1.
** Extrapolated value from Chart 1.

Group 2), indicated that 6-mercaptopurine and 5-FU decreased the virus titer in the drug-treated donors by 2.3 and 1.3 logs, respectively.

Chart 3B summarizes the results obtained with vincristine, 1,3-bis(2-chloroethyl)-1-nitrosourea, and methylglyoxalbisguanylatedrazine. Vincristine and 1,3-bis(2-chloroethyl)-1-nitrosourea were effective in retarding splenomegaly and inhibiting virus in treated donor animals by 2.6 and 1.4 logs, respectively (Table 1). Spleen weights of recipient animals given inoculations of dilutions of plasma from methylglyoxalbisguanylatedrazine-treated donors (Table 1, Group 5) were greater than those of the controls (Table 1, Group 2). The results suggest that this drug may have produced an increased virus yield.

Since the spleen weights of recipient mice given inoculations of the lowest dilution of treated-donor plasma (10^-5) were greater than those of normal mice (Charts 2A and B and 3A and B), it would appear that none of the drugs at the dose tested were capable of completely inhibiting virus.

DISCUSSION

The current study illustrates the use of the Rauscher virus for the detection and assay of anti-viral agents.

In adult BALB/c mice given inoculations intraperitoneally of log dilutions of Rauscher virus, the mean log spleen weight at an arbitrary time after inoculation was shown to be directly related to the dose of virus (Chart 1). Rowe (34), employing the Friend mouse leukemia virus, demonstrated that spleen weight at an arbitrary time after infection was a function of virus dose. A modification of the same procedure has been employed by Moloney* for the rapid in vivo assay of his leukemia virus. With this relationship, it was possible to employ the rate and degree of splenomegaly, observed in recipient

* J. B. Moloney, unpublished observations.
mice, as a measure of the comparative amounts of virus in control and drug-treated donor animals (Table 1, and Charts 2A and B and 3A and B). Although different preparations of virus may differ in "potency"—i.e., the rapidity with which a given number of infectious doses induce viremia and splenomegaly—this possibility is not a problem in this test system. Control virus-inoculated animals were bioassayed in recipient mice 5 and 9 days post-inoculation in each experiment to assess the virus titer in animals at the time of drug initiation (5th day) and at the time of sacrifice (9th day). The data presented in Charts 2A and B and 3A and B show the excellent reproducibility, from one experiment to another, of spleen weights in donor animals given inoculations of a standard dose of virus.

Mice receiving drug therapy displayed some loss of body weight. However, in most cases the mice regained weight rapidly. Although the loss in body weight was not extensive, it was considered possible that it may have affected the viremia. In any investigation of the selectivity of anti-viral chemotherapeutic agents it is desirable to determine to what extent nonspecific host toxicity, as reflected in reduction in food intake and weight loss of the host, may account for any observable anti-viral effect. An investigation is currently in progress and is designed to determine what effect body weight loss may have on the course of viremia.

The paucity of information concerning the anti-viral effect of drugs on oncogenic viruses in vivo makes it difficult to compare the results obtained with this test system with those of others. Mirand (28) reported that 6-MP was effective against the Friend virus in vitro and in vivo. Of the drugs tested in this study and that of Sugiraga (35), 6-MP and 6-thioguanine had a pronounced inhibitory effect, whereas 5-FU had a moderate inhibitory effect on the Friend virus leukemia. We previously reported increases in survival time of mice given inoculations of Rauscher virus following treatment with 6-MP from the 3d to the 7th day after infection (7). Levintow (26) reported that puromycin limited the synthesis of poliovirus RNA in infected HeLa cells. Anti-viral activity of thiocarbamates against the vaccinia (5), rabbit pox (3), and neurotropic vaccinia (2) viruses have been reported. However, the compound p-isopropyl benzaldehyde thiocarbamazone (NSC-9,936) did not show anti-viral activity against the Rauscher virus in the current study.

It is of interest that the treatment of mice with prednisone and methyl-GAG appeared to enhance the yield of virus. Similar observations with steroids have been reported for vaccinia and influenza B viruses. Kilbourne (25), employing influenza B virus and the chick embryo as his test system, reported that, when cortisone and related analogs were inoculated into the allantoic sac, an increased yield of virus was obtained. Similarly, Holden (19), in studies with hydrocortisone and vaccinia virus in tissue culture, observed a marked increase in the release of infective virus in comparison with cells cultivated in the absence of the steroid. Kásosvá (24) inoculated mice extraneurally with encephalitis virus and observed that cortisone treatment resulted in an increased amount of virus in blood, spleen, and brain. The apparent increased yield of Rauscher virus in mice treated with prednisone and methylglyoxalbisguanylhydrazone in the current study may not be significant. Detailed studies are currently in progress attempting to quantitate the increase in virus yield by these and other drugs in vivo.

The inability of viruses to replicate extracellulary provides one of the basic problems in anti-viral chemotherapy. Chemotherapeutic inactivation of extracellular virus (in vitro and in vivo) without concomitant selective inhibition of infected cells virtually guarantees continuation of the virus and its induced disease. The problem is especially acute in diseases caused by viruses, the synthesis and maturation cycles of which do not include lysis of the infected cell. A further difficulty is the finding that oncogenic viruses reproduce not only in their induced tumor cells, which may be amenable to therapy by anti-tumor drugs, but also within nonmalignant reservoir cells. The synthesis of avian leukosis virus in pancreatic acinar cells of normal chicks (41) and of several murine leukemia viruses in megakaryocytes of leukemic mice (11-13) suggests that, to control a virus-induced neoplasm, a drug or combination of drugs must be effective against the tumor cell, the virus, and against cells which synthesize the virus. Contributions to the solution of these problems are dependent on the development and application of host-virus model systems in which the complex interactions of virus, host, and drug may be quantitated. The current study represents one attempt to test and evaluate the relative capacities of therapeutic agents to modify the virus, its synthesis, and its induced neoplasm.

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