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# Combination Chemotherapy of Rauscher Leukemia and Ascites Tumors by Narcissus Alkaloid with Standard Drugs and Effect on Cellular Immunity[1] (38896)

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In an antiviral screening program with 400 medicinal plants of the Pacific area, it was found that one of the narcissus alkaloids (residual alkaloid A-2) had a remarkable prolongation effect on the life span of advanced Rauscher leukemic mice (1–3). The alkaloid is one of the few potent inhibitors of viral reverse transcriptase effective at the animal level (4) and has been identified as pretazettine. The alkaloid does not inhibit the production of humoral antibody against sheep red cells nor the induction of interferon by poly (I:C) in leukemic mice (1). The rational combination of the alkaloid (antiviral DNA) with cyclophosphamide or 6-mercaptopurine (anticellular DNA) shows synergistic activity (1). We report now combination effects of the narcissus alkaloid with other classified standard anticancer drugs against both Rauscher leukemia and transplantable ascites tumors, and also the effects on cell-mediated immunity estimated by lymphocyte reactivity against phytohemagglutinin (PHA) *in vitro* and by allogeneic tumor rejection in mice.

*Materials and Methods. Preparation of the narcissus alkaloid and other drugs.* Methods of extraction of the total alkaloidal fraction and of the crude residual alkaloid from the bulbs of *Narcissus tazetta* L. have been described (5). The residual alkaloid A-2 is a partially purified grade, soluble in cold water, and obtained by repeated washing and drying with water. The residual alkaloid A-3 is a chromatographically pure alkaloid isolated from A-2 grade by passing through a silica gel column with chloroform and then with a mixture of chloroform and methanol (9:1). Alkaloid A-3 is the main component (ca. 80%) of alkaloid A-2 and has now been identified as pretazettine by Drs. Irie, Tani, and Uyeo of Kyoto University (personal communication). Figure 1 is the structure reported by Dr. Wildman of Iowa University (6). Because of the poor yield of A-3 grade by present methods, we used the A-2 grade for the therapeutic experiments in mice. Methotrexate, 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) and streptonigrin were obtained from Dr. Chirigos of NIH, and the other drugs were purchased from commercial sources.

*Rauscher viral leukemia.* An inoculum of 0.2 ml of a 1:40 dilution of leukemic plasma (days 30–40) was injected intraperitoneally (ip) into 6- to 8-wk-old BALB/c mice, either male or female. Treatment with optimal dose of the drugs was started subcutaneously (sc) 16–18 days after infection when 70–100% of the mice showed clearly palpable spleens. All drugs, singly or combined, were injected every other day and continued until 36–46 days. The doses and the periods were chosen to avoid any apparent toxicity as indicated by general weakness, ruffled hair, weak motions, marked loss of body weight, or marked decrease of intake of food and water.

*Transplantable tumor systems.* Ehrlich ascites tumor cells have been passaged ip in a random strain of Swiss albino mice for many years. The ascites line of MCDV-12 leukemia cells originated from a Rauscher virus-inoculated BALB/c male mouse was supplied by Dr. Chirigos, and carried in BALB/c inbred mice for 4 yr by Dr. Yoder, University of Hawaii. At present, no infective Rauscher virus can be detected in the cells.

*Spleen cell culture for PHA reactivity.* Using the method of El-Arini and Osoba (7) suspensions of spleen cells were made in RPMI-1640 medium with 5% heat-inacti-

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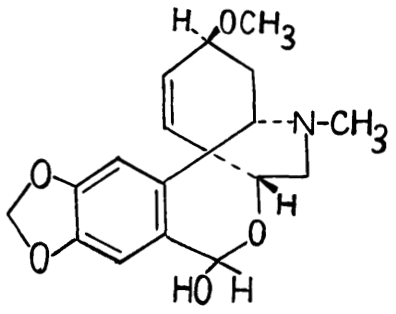

FIG. 1. Pretazettine (alkaloid A-3).

vated human serum, in lieu of fetal calf serum to prevent a nonspecific stimulation (8) as follows: Each spleen was minced by scissors into an almost viscous state without fluid, and suspended in 10 ml of the medium by mild pipetting. After the large clumps had settled, the supernatant was pushed through a stainless-steel 100-mesh screen connected with a syringe, then centrifuged, resuspended in the fresh medium, and a cell count made. The 5 x $10^6$/3 ml spleen cells were incubated in screw-capped Falcon plastic tubes (16 × 125 mm) for 48 hr at 37° in a $CO_2$-incubator (5% $CO_2$). The 0.1 ml of various dilutions of phytohemagglutinin (PHA-P, Difco Lab.), freshly made, was added to each tube just before incubation. Six hours before harvesting the cells, 0.3 μCi of $^{14}$C-thymidine (ICN Isotope & Nuclear Div., 58.5 mCi/mmole) were added to each tube. The cells were collected on a Whatman filter paper (3 MM) and washed twice in cold saline, 5% trichloracetic acid, 50% alcohol, absolute methanol, and ether, respectively. The dried filter was then placed in a counting vial containing 10 ml of toluene-base fluid and counted for 10 minutes with a Beckman beta-II liquid scintillation counter.

*Whole blood cultures for PHA reactivity.* Cell suspensions for lymphocytic activity were prepared by adding heparinized whole blood, collected by heart puncture, to 30 vol of RPMI-1640 medium not supplemented by any serum according to the method of Pauly and Sokal (9). The 3-ml aliquots of the diluted blood were distributed to Falcon plastic tubes (16 × 125 mm), 0.1 ml of various dilutions of PHA-P added, and the loosely capped tubes incubated at 37° in an upright position for 5 days in a $CO_2$-incubator. Twenty-four hours before termination of culture, 0.3 μCi of $^{14}$C-thymidine were added to each tube. For harvesting, 3 vol of cold 3% acetic acid were added to lyse erythrocytes; the resulting leukocyte suspension was centrifuged and the cells collected on a Whatman filter paper. The amount of activity in the 5% trichloracetic acid precipitates was determined by the method described above.

*Allogeneic tumor-rejection system.* Hybrid BALB/c Fl mice (BALB/c inbred male X Swiss albino female) were inoculated ip with 5 × $10^5$ cells of MCDV-12 leukemia, a syngeneic cell of BALB/c mice that had been passaged in the inbred mice. About 60% of the Fl mice were expected to reject the cells and survive, although temporary growth was observed in about 50% of the survivors. Pretreatment of the hybrid mice with potent immunosuppressive drugs is, therefore, expected to increase the death rate by allowing the progression of tumor growth.

*Results. Combination effect of narcissus alkaloid with standard drugs on survival of mice with established Rauscher leukemia.* Currently, combination chemotherapy with agents having different action mechanisms has been required for the best response in advanced animal or human cancers and is becoming a standard therapeutic approach (10). Therefore, as one of the essential steps to clinical application, we have tested a combination spectrum of the narcissus alkaloid with standard anticancer drugs selected by their type of action against murine viral leukemia and transplantable tumors. The drugs used were: alkylating agents (cyclophosphamide, BCNU), DNA-binding antibiotics (daunorubicin, dactinomycin), antimetabolites (methotrexate, 6-MP, 5-FU, 5-azacytidine) and alkaloids (vincristine, emetine). Table I shows the effect of the combined drugs when administered continuously, every other day for 20–30 days, against advanced Rauscher leukemia. Five drugs (BCNU, cyclophosphamide, dactinomycin, 6-MP, and 5-azacytidine) combined with narcissus alkaloid showed significant ($P < 0.01$) increases in prolonging the life

TABLE I. COMBINATION EFFECT OF NARCISSUS ALKALOID WITH STANDARD DRUGS ON SURVIVAL OF MICE WITH ESTABLISHED RAUSCHER LEUKEMIA.[a]

| Expt no. | Agent | Dose (mg/kg) | Median survival time (MST) (day and range) | Prolongation of MST | | P |
|---|---|---|---|---|---|---|
| | | | | Over control | Over alk. A-2 | |
| E1 | Control (each 70 mice) | | 36 (24–47)[b] | | | |
| | BCNU | 2.5 | 50 (26–62) | 39% | | |
| | Alkaloid A-2 | 25.0 | 55 (29–78) | 53 | | |
| | Mixture | | 73 (39–90) | 103 | 94% | <0.01 |
| E2 | Control (each 40 mice) | | 38 (23–51) | | | |
| | Cyclophosphamide | 5.0 | 58 (28–71) | 53 | | |
| | Alkaloid A-2 | 25.0 | 81 (28–105) | 113 | | |
| | Mixture | | 114 (33–126) | 200 | 77 | <0.01 |
| E3 | Control (each 25 mice) | | 34 (27–43) | | | |
| | Daunorubicin | 0.25 | 41 (27–51) | 21 | | |
| | Alkaloid A-2 | 25.0 | 64 (29–82) | 88 | | |
| | Mixture | | 60 (29–84) | 77 | 0 | |
| E4 | Control (each 20 mice) | | 40 (24–58) | | | |
| | Dactinomycin | 0.05 | 56 (27–70) | 40 | | |
| | Alkaloid A-2 | 12.5 | 57 (30–69) | 43 | | |
| | Mixture | | 80 (37–95) | 100 | 132 | <0.01 |
| E5 | Control (each 30 mice) | | 33 (24–42) | | | |
| | Methotrexate | 1.5 | 47 (30–56) | 42 | | |
| | Alkaloid A-2 | 25.0 | 67 (35–79) | 103 | | |
| | Mixture | | 66 (34–75) | 100 | 0 | |
| E6 | Control (each 30 mice) | | 40 (24–58) | | | |
| | 6-MP | 2.5 | 56 (26–69) | 40 | | |
| | Alkaloid A-2 | 25.0 | 75 (32–105) | 88 | | |
| | Mixture | | 101 (39–124) | 152 | 73 | <0.01 |
| E7 | Control (each 20 mice) | | 31 (23–42) | | | |
| | 5-FU | 5.0 | 37 (26–45) | 19 | | |
| | Alkaloid A-2 | 12.5 | 48 (30–66) | 55 | | |
| | Mixture | | 46 (30–70) | 49 | 0 | |
| E8 | Control (each 20 mice) | | 42 (28–54) | | | |
| | 5-Azacytidine | 3.0 | 57 (31–68) | 37 | | |
| | Alkaloid A-2 | 25.0 | 71 (40–83) | 70 | | |
| | Mixture | | 101 (42–127) | 143 | 103 | <0.01 |
| E9 | Control (each 20 mice) | | 32 (23–39) | | | |
| | Vincristine | 0.04 | 39 (23–44) | 22 | | |
| | Alkaloid A-2 | 25.0 | 58 (33–72) | 81 | | |
| | Mixture | | 52 (31–69) | 63 | 0 | |
| E10 | Control (each 20 mice) | | 36 (26–45) | | | |
| | Emetine | 4.0 | 45 (25–51) | 25 | | |
| | Alkaloid A-2 | 25.0 | 78 (35–108) | 117 | | |
| | Mixture | | 70 (34–98) | 94 | 0 | |

[a] Treatment with the optimal dose of each agent or the same dose of the mixture was started on Days 16–18 and continued every other day until Days 36–46.

[b] Day of the first death to day of the last death during the observation periods of 90–130 days.

span of the leukemic mice compared with single-drug administration. The other five drugs (daunorubicin, methotrexate, 5-FU, vincristine, and emetine) showed no beneficial effect in combination with the narcissus alkaloid. The amount of the standard drugs used was the maximum nontoxic doses (MNTD) and the amount of the narcissus alkaloid was one half of the MNTD for the long-term schedules. Mixtures of the same

TABLE II. COMBINATION EFFECT OF NARCISSUS ALKALOID WITH STANDARD DRUGS ON EHRLICH CARCINOMA CELLS IN MICE.[a]

| | Maximum nontoxic dose/kg (mg) | Median survival time (MST) (days and range) | Prolongation of MST over control (%) | Survivals/total no. of mice[b] (%) | |
|---|---|---|---|---|---|
| Control | | 16.3 (16–18)[c] | | 0/141 | 0 |
| Alkaloid A-2 | 50 | 24 (22–32) | 47 | 20/89 | 22 |
| BCNU | 10 | 27.3 (25–30) | 52 | 7/49 | 14 |
| BCNU + A-2 | | >44.3 (35–50) | >148 | 29/40 | 73 |
| Cyclophosphamide | 200 | 34.2 (27–39) | 94 | 3/39 | 8 |
| Cyclo. + A-2 | | >60 (50–70) | >241 | 12/18 | 67 |
| Daunorubicin | 0.5 | 19.5 (16–22) | 26 | 2/25 | 8 |
| Dauno. + A-2 | | >28.5 (25–34) | > 84 | 15/25 | 60 |
| Dactinomycin | 0.2 | 22.5 (20–25) | 36 | 1/18 | 6 |
| Dact. + A-2 | | >31.3 (28–40) | > 89 | 6/18 | 33 |
| Methotrexate | 15 | 21.8 (19–25) | 28 | 0/18 | 0 |
| Metho. + A-2 | | 24 (20–28) | 40 | 4/12 | 33 |
| 6-MP | 90 | 21.5 (18–25) | 23 | 2/12 | 17 |
| 6-MP + A-2 | | 24.5 (20–29) | 40 | 2/12 | 17 |
| 5-FU | 20 | 20.8 (18–24) | 26 | 2/14 | 15 |
| 5-FU + A-2 | | 24 (21–27) | 45 | 3/12 | 25 |
| 5-Azacytidine | 15 | 18.3 (17–20) | 8 | 0/18 | 0 |
| 5-Aza. + A-2 | | 23 (20–26) | 35 | 2/18 | 11 |
| Vincristine | 0.5 | 21 (19–23) | 27 | 0/14 | 0 |
| Vinc. + A-2 | | 23 (19–27) | 35 | 0/12 | 0 |
| Emetine | 15 | 18 (16–20) | 13 | 0/12 | 0 |
| Emetine + A-2 | | 24.5 (20–29) | 50 | 2/12 | 17 |

[a] The $10^5$ cells were inoculated ip. Only 1 ip injection of the standard drugs on Day 1 (priming) was followed by the seven daily ip injections of Alkaloid A-2 started on Day 1 in the combination group. Other groups were given only one injection of the drug except the A-2 group given seven daily injections.

[b] Survivals without appearance of ascites on or over Day 30.

[c] Average and the range of MST of each experiment which was composed of 6–10 mice.

doses did not show any apparent increase in toxicity during the periods of administration.

*Combination effect of narcissus alkaloid with standard drugs against transplantable tumors.* Standard cytotoxic drugs cannot be administered repeatedly without toxicity (11) and thus do not eradicate the residual tumor cells. Therefore, the combination schedule of the one priming heavy dose of the standard drugs followed by the multiple (daily) doses of the narcissus alkaloid A-2 has been adopted to eradicate the tumor cells of Ehrlich carcinoma and MCDV-12 leukemia. Table II shows the results. A remarkable synergistic effect is seen when the narcissus alkaloid is combined with the alkylating and DNA-binding agents (BCNU, cyclophosphamide, daunorubicin and dactinomycin), while little beneficial effect is obtained by combination with other groups (antimetabolites and alkaloids) of standard drugs. Similarly, as shown in Table III, the positive effect against MCDV-12 leukemia appeared limited to a combination with the alkylating and DNA-binding agents. Combinations of the narcissus alkaloid with other groups of standard drugs were neigher beneficial nor harmful so the data were omitted. The narcissus alkaloid, daunorubicin, and dactinomycin showed weak activity (2-day prolongation of life span: $P < 0.05$) and BCNU and cyclophosphamide were remarkably active ($P < 0.01$) when administered singly. The narcissus alkaloid combined with cyclophosphamide was especially effective since 50% of the mice survived for over 90 days without apparent tumor growth.

*Effect of narcissus alkaloid on PHA reactivity of spleen and whole blood cells.* In cancer chemotherapy, the immunosuppres-

TABLE III. COMBINATION EFFECT OF NARCISSUS ALKALOID WITH STANDARD DRUGS ON MCDV-12 LEUKEMIA CELLS IN MICE.[a]

| | Maximum nontoxic dose/kg (mg) | Median survival time (MST) (days and range) | Prolongation of MST over control (%) | (P) | Survival/ total no. of mice | (%) | (P) |
|---|---|---|---|---|---|---|---|
| Control | | 9.3 ( 9–10) | | <0.05 | 0/95 | 0 | |
| Alkaloid A-2 | 50 | 11.5 (10–13) | 24 | | 2/30 | 7 | <0.05 |
| BCNU | 10 | 16 (13–19) | 72 | | 4/30 | 13 | |
| BCNU + A-2 | | 22.8 (20–26) | 145 | <0.05 | 3/18 | 17 | <0.05 |
| Cyclophosphamide | 200 | 23.8 (20–27) | 156 | | 5/29 | 17 | <0.01 |
| Cyclo. + A-2 | | >40 (36–45) | >330 | <0.05 | 9/18 | 50 | |
| Daunorubicin | 0.5 | 11.8 (10–13) | 27 | | 2/24 | 8 | |
| Dauno. + A-2 | | 19.3 (17–21) | 108 | <0.01 | 3/18 | 17 | <0.05 |
| Dactinomycin | 0.2 | 11 (10–12) | 22 | | 0/12 | 0 | |
| Dact. + A-2 | | 13 (11–15) | 44 | <0.05 | 0/12 | 0 | |

[a] The $10^5$ cells were inoculated ip to BALB/c inbred mice. The treatment schedule was the same as shown in Table II.

TABLE IV. PHYTOHEMAGGLUTININ REACTIVITY OF SPLEEN AND WHOLE BLOOD CELLS FROM MICE PRETREATED WITH NARCISSUS ALKALOID.[a]

| Expt no. and cell type | | Agent | Pretreatment dose schedule | PHA reactivity (ratio and cpm)[b] PHA 1:20 | PHA 1:40 | PHA 1:80 | no PHA |
|---|---|---|---|---|---|---|---|
| E1 | Whole | Control | | 19 (461) | 5 (126) | 2 ( 55) | 1 (24) |
| | blood | Alkaloid A-2 | 1 mg, q2d × 10[c] | 18 (827) | 4 (169) | 2 (109) | 1 (46) |
| E2 | Whole | Control | | 7 (473) | 13 (905) | 2 (145) | 1 (71) |
| | blood | Alkaloid A-2 | 4 mg × 1 (−24 h)[d] | 7 (473) | 11 (634) | 8 (497) | 1 (60) |
| E3 | Whole | Control | | 20 (754) | 8 (320) | 5 (188) | 1 (38) |
| | blood | Alkaloid A-2 | 6 mg × 1 (−6 h)[c] | 17 (1,261) | 9 (628) | 4 (303) | 1 (73) |
| E4 | Spleen | Control | | 9 (10,078) | 9 (9,678) | | 1 (1,103) |
| | | Alkaloid A-2 | 1 mg, q2d × 10[c] | 7 ( 8,592) | 8 (9,990) | | 1 (1,226) |
| E5 | Spleen | Control | | | 11 (12,394) | 8 (8,751) | 1 (1,146) |
| | | Alkaloid A-2 | 4 mg × 1 (−24 h)[d] | | 14 (13,876) | 10 (10,215) | 1 (1,024) |
| E6 | Spleen | Control | | 9 (21,973) | 7 (17,207) | | 1 (2,471) |
| | | Alkaloid A-2 | 6 mg × 1 (−6 h)[d] | 12 (25,852) | 9 (19,483) | | 1 (2,085) |

[a] The 0.1 ml of the diluted PHA was added in 3 ml of culture medium which contained 0.1 ml of whole blood or 5 × $10^6$ spleen cells per tube, then incubated for 3 days (spleen) or 4 days (whole blood). The 0.3 μCi of $^{14}$C-thymidine was added 6 hr (spleen) or 24 hr (whole blood) before termination.

[b] Counting efficiency was checked and different cpms are comparable ones as far as efficiency is concerned. The ratio is the degree of stimulation compared with controls (no PHA).

[c] Mice were treated with 1-mg dose (50 mg/kg) of A-2, sc, every other day, total 10 injections until 1 day before the blood and spleen harvested.

[d] Mice were treated with a single sc injection of A-2 24 hr or 6 hr before the preparation of the cells.

sive activity of various anticancer drugs is one of the most unfavorable side effects (12). The narcissus alkaloid at the therapeutic dose was not inhibitory to humoral antibody production (1). We have tested the alkaloid for its possible inhibitory effect on cellular immunity. It has been established by many investigators that PHA activates only the lymphocytes associated with cellular immunity (13). Normal mice were pretreated with 10 multiple optimal doses (1 mg, sc, every other day) or one subtoxic dose (4 or 6 mg) of narcissus alkaloid, then whole blood and spleens were collected 6 hr or 24 hr after the last injection, and the PHA reactivity of the cells tested *in vitro*. As shown in Table IV, there are no significant differences in the reactivity of the blood or spleen cells between the control and the alkaloid-treated mice. This shows that the alkaloid has no adverse effect on the PHA reactivity of the lymphocytes of the pretreated mice.

*Effect of narcissus alkaloid on allogeneic tumor rejection.* It is well known that the

TABLE V. EFFECT OF NARCISSUS ALKALOID AND STANDARD DRUGS ON ALLOGENEIC TUMOR REJECTION (MCDV-12 AND BALB/c F1).[a]

| Expt no. | Agent | Pretreatment dose/mouse | Survival | | Tumor growth[b] | |
|---|---|---|---|---|---|---|
| | | | Total | | Total | |
| | | | No. | % | No. | % |
| E1 | Control | | 8/13 | 62 | 7/13 | 54 |
| | Cyclophosphamide | 250 μg, q2d × 10[c] | 3/13 | 23 | 12/13 | 92 |
| | Alkaloid A-2 | 1 mg, q2d × 10 | 9/13 | 69 | 7/13 | 54 |
| E2 | Control | | 6/10 | 60 | 5/10 | 50 |
| | Cyclophosphamide | 3 mg × 1[d] | 3/10 | 30 | 9/10 | 90 |
| | Cyclophosphamide | 4 mg × 1 | 0/10 | 0 | 10/10 | 100 |
| | Alkaloid A-2 | 4 mg × 1 | 6/10 | 60 | 6/10 | 60 |
| | Alkaloid A-2 | 6 mg × 1 | 5/10 | 50 | 5/10 | 50 |
| | Streptonigrin | 5 μg × 1 | 5/8 | 63 | 4/8 | 50 |
| | Dactinomycin | 4 μg × 1 | 2/8 | 25 | 7/8 | 88 |
| E3 | Control | | 6/10 | 60 | 5/10 | 50 |
| | 6-MP | 3 mg × 1 | 6/10 | 60 | 8/10 | 50 |
| | Daunorubicin | 20 μg × 1 | 3/10 | 30 | 9/10 | 90 |
| | Vincristine | 20 μg × 1 | 5/10 | 50 | 9/10 | 90 |
| | Alkaloid A-2 | 4 mg × 1 | 5/10 | 50 | 5/10 | 50 |
| E4 | Control | | 7/10 | 70 | 4/10 | 40 |
| | BCNU | 0.4 mg × 1 | 2/10 | 20 | 9/10 | 90 |
| | Methotrexate | 80 μg × 1 | 4/10 | 40 | 8/10 | 80 |
| | 6-MP | 3 mg × 1 | 7/10 | 70 | 8/10 | 80 |
| | Streptonigrin | 10 μg × 1 | 7/10 | 70 | 5/10 | 50 |
| | Alkaloid A-2 | 6 mg × 1 | 6/10 | 60 | 5/10 | 50 |

[a] Hybrid F1 mice (BALB/c male X Swiss albino female) were inoculated ip with 500,000 cells of MCDV-12 leukemia, syngeneic cells of BALB/c.

[b] Included the temporal and progressive growths of the tumor judged by the development of the ascites.

[c] Mice were treated with the dose, sc, every other day, 10 injections until 1 day before tumor inoculation.

[d] Mice were treated with a single sc injection of the dose 1 day before tumor inoculation.

rejection of transplanted tumors is due primarily to the cellular immune system (14). In a system of MCDV-12 leukemia cells and BALB/c F1 mice, about 60% of the mice inoculated with the tumor cells can survive, although tumor growth is observed temporarily in about 50% of the survivors. We have also tested the possible inhibitory effect of the alkaloid on this rejection system by comparison with some standard drugs. Table V shows the results. In Experiment 1, mice have been treated sc with small but therapeutic doses of the agents every other day for 20 days before the tumor cell inoculation. In the cyclophosphamide-treated group 12 of 13 mice (92%) showed tumor growth (appearance of ascites), 10 of the 13 mice (77%) died of progressive tumor growth, while 3 of 13 (23%) rejected the tumor and survived. In the alkaloid-treated group, 54% showed tumor growth, 31% died, and 69% finally survived; just as the control group. In Expts 2–4, mice were treated sc with a single heavy therapeutic dose of each agent 1 day before tumor inoculation. All of the four alkylating and DNA-binding agents enhanced the tumor growth thus reducing the survival rate ($<30\%$: $P < 0.01$). The other standard drugs (6-MP, methotrexate, vincristine) enhanced the tumor growth temporarily but resulted in a final rejection, while the narcissus alkaloid and streptonigrin, both new potent inhibitors of reverse transcriptase (4, 15), showed no significant effect on this rejection system.

*Discussion.* The narcissus residual alkaloid is one of the few potent inhibitors of reverse transcriptase (4) therapeutically effective against Rauscher leukemia in mice and strongly inhibits the growth of Rauscher virus in cell cultures (3). Its antiviral leukemic activity seems to be superior to other standard drugs in view of the prolonged effect on the life span. This is the most important criterion in evaluating anticancer agents, the result of the over-all effects involving their specific activity and general toxicity.

Combination chemotherapy is now a standard therapeutic approach to obtain the best response in advanced cancer (10). We expect to use the narcissus alkaloid first as a supplemental agent to the standard drugs against human acute leukemia which possesses the reverse transcriptase (16). Therefore, we have determined a rough spectrum of the standard drugs with which the narcissus alkaloid should be combined in order to provide a better response. The spectrum seems to be limited to the alkylating and DNA-binding agents against transplantable tumors, although 6-MP and 5-azacytidine are also available for Rauscher leukemia.

The alkaloid does not inhibit humoral antibody production nor interferon induction (1). Many standard drugs possess strong or moderate immunosuppressive activity not only on humoral but also on cellular immunity which plays an important role in preventing the progression of tumor growth (12, 14). The alkaloid has now been found to have no adverse effects on the cellular immunity as demonstrated by tests of PHA reactivity and allogeneic tumor rejection. We have confirmed that most of the standard cytotoxic drugs used for comparison show progressive or temporary suppression of the rejection mechanisms. Streptonigrin, a new potent inhibitor of reverse transcriptase reported by Dr. Chirigos (15), also did not affect the allogeneic rejection and our preliminary data confirm the reported activity on Rauscher leukemic mice. Concerning the chemistry of the narcissus residual alkaloid, the A-2 grade which was used in our *in vivo* experiments has been found to contain pretazettine in large amounts (ca. 80%) as well as other alkaloids (tazettine, lycorine, and pseudolycorine). The alkaloid A-3, chromatographically purified grade, has been identified as pretazettine (6) by Dr. Irie *et al.* of Kyoto University and confirmed by us by comparing with a standard sample of pretazettine supplied by Dr. W. C. Wildman of Iowa University. At a concentration of 2.5 μg/ml, the A-3 inhibited Rauscher virus growth in cultures (3) and at 60 μg/ml, it was inhibitory (50%) to the reverse transcriptase of avian myeloblastosis virus (personal communication from Dr. Chirigos of NIH) which was equivalent to 2 mg/ml of the crude residual alkaloid. The *in vivo* anti-Rauscher leukemia tests with the A-3 are now in progress.

*Summary.* The therapeutic activity of the narcissus residual alkaloid A-2 against Rauscher leukemia has been compared with 10 standard anticancer drugs, and synergistic or additive combination pairs have been selected using a viral leukemia and two transplantable tumor systems. An increased beneficial effect has been demonstrated by a combination of the alkylating and DNA-binding agents and the alkaloid against the three malignant tumors, while a beneficial effect by combining the alkaloid and the antimetabolites (either 6-MP or 5-azacytidine) was seen only against the viral leukemia. The alkaloid has no suppressive activity against cellular immunity as tested by PHA reactivity and allogeneic tumor rejection systems.

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