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Synergistic Suppression of the Hyperacute Form of Experimental Allergic Encephalomyelitis by Tilorone and Cycloleucine[1] (39956)

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Combinations of drugs have been used extensively for chemotherapy of cancer, and many instances of synergistic relationships have been found. The criteria for a synergistic relationship have been critically and clearly reviewed by Berenbaum (1). He found only a few valid reports of synergy among immunosuppressive drugs. There have been very few efforts to develop drug combinations for immunosuppression of the experimental autoimmune disorders, although these disorders are useful models for a number of important diseases of man. This is probably due to the fact that disease states are more difficult to quantitate than antibody levels. Experimental allergic encephalomyelitis (EAE), a prototype of cell-mediated autoimmune diseases, can be produced in a hyperacute form (2, 3) which is more uniform and reproducible than most other immunopathological conditions. With the aid of this model, we have surveyed the immunosuppressive effects of combinations of antilymphocyte serum, cyclophosphamide (an alkylating agent) (4), cycloleucine (an unnatural amino acid) (5, 6), tilorone (a bis-basic substituted fluorene-9-one compound that depletes T lymphocytes) (7, 8), and other drugs. Many combinations gave augmented immunosuppressive effects, and the interaction of cycloleucine and tilorone was proven to be synergistic.

Methods. Female Lewis rats, 150-200 g, from Microbiological Associates, Inc., Bethesda, Md., were maintained in hanging wire cages on Purina laboratory chow and tap water. After at least 1 week of acclimatization, EAE was induced by intradermal footpad inoculation of 0.05 ml of a water-in-oil emulsion of 40% (wet weight/volume) guinea pig spinal cord homogenate in an equal volume of Freund's adjuvant (8.5 parts of Bayol F, 1.5 parts of Arlacel A, 4 mg/ml of killed human tubercle bacilli). Pertussis vaccine concentrate, approximately 20 billion organisms in 0.1 ml, was injected at the same time into the dorsum of the same foot in order to produce the hyperacute form of EAE. Homogenates and emulsions were prepared by cycling between two syringes connected by a double-hubbed needle.

Scoring of hyperacute EAE was based on time of onset of earliest clinical signs (weakness, flaccid tail). Severity of signs is not included in the tables because almost all rats eventually became paralyzed even when treatment had delayed the onset of signs and prevented death.

Single doses of serum or drug freshly prepared in saline were given on the day of immunization (DO). Certain groups of rats were given a second drug on the same day or 1 or 4 days later. The doses were: antilymphocyte serum (ALS, rabbit or horse anti-rat), 5 ml/kg ip; cyclophosphamide (CY), 50 mg/kg sc; cycloleucine (CL), 150 mg/kg orally; tilorone hydrochloride (T), 200 mg/kg orally. Control rats were given saline orally or sc.

Rats were weighed weekly. Four weeks after inoculation, they were killed by exsanguination from neck vessels while under ether anesthesia. Thymus and spleen were weighed fresh and then fixed with entire spinal cord in Bouin's fluid. Paraffin-embedded sections were cut and stained with hematoxylin and eosin.

Results. Signs of EAE became obvious 7 or 8 days after immunization in almost all control rats, and all became paralyzed and died, usually 9 to 11 days after inoculation. One dose of any one of the four agents delayed onset from 1 to 3 days (Table I). Treatment with two of the agents produced a considerably greater delay in onset in

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TABLE I. SUPPRESSION OF HYPERACUTE EAE BY PAIRS OF DRUGS.

Treatment[a]			EAE	
D0	D1	D4	Onset[b]	Mortality[c]
ALS	–	–	9.6 ± 0.9*	18/20
ALS + CY	–	–	14.8 ± 1.7	2/5
ALS	CY	–	16.6 ± 2.3	1/5
ALS	–	CY	13.8 ± 1.3	2/5
ALS + CL	–	–	17.4 ± 0.8	0/5
ALS	–	CL	14.4 ± 1.6	0/5
ALS + T	–	–	16.5 ± 2.5	3/8
ALS	T	–	14.8 ± 0.8	0/5
ALS	–	T	12.4 ± 1.4	5/5
CY	–	–	9.4 ± 0.9*	8/10
CY	ALS	–	21.3 ± 0.4	0/5
CY	–	ALS	13.4 ± 0.5	0/5
CY + CL	–	–	16.0 ± 2.2	0/3
CY	–	CL	14.0 ± 1.4	0/4
CY + T	–	–	11.3 ± 0.5	3/5
CY	T	–	12.3 ± 0.4	4/4
CY	–	T	10.4 ± 0.5	5/5
CL	–	–	10.6 ± 1.4*	2/15
CL	–	ALS	16.2 ± 3.6	0/4
CL	–	CY	18.7 ± 1.3	0/3
CL + T	–	–	19.0 ± 2.6	1/10
CL	–	T	15.8 ± 0.9	0/4
T	–	–	9.4 ± 0.8*	14/30
T	ALS	–	12.1 ± 2.6	3/10
T	–	ALS	9.6 ± 0.5	5/5
T	CY	–	14.7 ± 1.9	1/10
T	–	CY	13.6 ± 0.5	2/5
T	CL	–	20.3 ± 0.5	0/3
T	–	CL	21.0 ± 1.9	0/10
d	–	–	7.4 ± 0.5	49/50

[a] ALS, antilymphocyte serum; CY, cyclophosphamide; CL, cycloleucine; T, tilorone; d = pooled saline-treated control rats from several experiments. D0 = day of immunization.

[b] Average of days on which earliest sign appeared, ± SD.

[c] Numerator, number of rats that died; denominator, total number of rats.

* Onsets following treatment with a single drug were compared to onsets after combinations of drugs by Student t test. All these differences were highly significant ($P < 0.001$) except the last combination under CY and the second combination under T, which were not significant.

almost all instances. The effect of some of the combinations was dependent on the schedule of administration. For example, tilorone given before cyclophosphamide or cycloleucine produced greater delays in onset than the reverse sequence. The poor results with the reverse sequence may be explained by the fact that many drugs create stress which interferes with the ability of subsequently administered tilorone to deplete T lymphocytes (8). Combinations involving ALS appeared to be schedule dependent, but not in any simple or explicable pattern.

The delay in onset caused by combinations of drugs was usually greater (and sometimes much greater) than the sum of delays that could be attributed to each agent alone. However, this fact is not sufficient to prove a synergistic relationship because the shape of the dose-response curve is not known (1). The data of Table I are useful for screening and to detect schedule dependency, not for proof of synergy. From Table I we selected cycloleucine and tilorone for a study of synergy. A single treatment with the drugs was given at full, one-half, or one-fourth the usual dose levels (9) (Table II). Individually, neither drug at half-dose levels produced more than a slight delay in onset. However, the combination of both drugs at half-dose levels gave far better results than full doses of either drug alone. Finally, the combination of both drugs at quarter-dose levels was approximately equal to full doses of either drug alone and definitely better than half-doses of either drug alone.

Despite the efficacy of tilorone combined with cycloleucine, the effects of the single treatment eventually disappeared. On the other hand, the inoculum persisted in feet and draining lymph nodes. Therefore, the synergy was manifested as a delay rather than prevention of disease. Almost every treated rat eventually developed clinical

TABLE II. SYNERGISTIC SUPPRESSION OF HYPERACUTE EAE BY COMBINATIONS OF CYCLOLEUCINE (CL) AND TILORONE (T).

Drug(s) on D0[a]	Delay in onset[b]	P value[c]
CL	3.0 ± 1.1	<0.005
T	2.6 ± 0.5	<0.001
1/2 CL + 1/2 T	6.8 ± 1.9	–
1/2 CL	1.0 ± 0	<0.005
1/2 T	2.0 ± 0	<0.02
1/4 CL + 1/4 T	3.8 ± 1.2	–

[a] Doses as specified in Methods, except where fractional doses are indicated.

[b] Average day of onset minus 7.0 (average onset in concomitant controls), ± SD (5 rats per group, except 10 for 1/2 CL + 1/2 T).

[c] Statistical significance of differences in delay caused by 1/2 CL + 1/2 T compared to CL or to T, and 1/4 CL + 1/4 T compared to 1/2 CL or to 1/2 T.

signs which usually progressed to paralysis. In an additional experiment, 10 rats that had been treated with full doses of *both* drugs on the day of inoculation were given a second identical treatment 13 days later. The second treatment gave further benefit in that three rats failed to develop signs during a 34 day observation period; the remaining seven rats did not have an onset until 24–29 days after inoculation (average 26.1), and the signs progressed to paralysis only in four rats.

Cyclophosphamide or tilorone on Day 0 were tried in a few other combinations (not included in the tables). Busulfan (10 mg/kg ip on Day 0 or 4) and indomethacin and phenylbutazone (3 and 100 mg/kg, respectively, orally, on Day 0, repeated on the next 4 days) failed to increase the modest suppressive effects of either cyclophosphamide or tilorone. Hydrocortisone (50 or 250 mg/kg sc on Day 0 or 4) enhanced the effects of either cyclophosphamide or tilorone, but only to a slight degree.

Discussion. Combinations of drugs have been used very little for suppression of EAE. Pellet *et al.* (10) claimed potentiation between cortisone and azathioprine, but perusal of their data raises questions about the adequacy of the controls. Vogel and Calabresi (11) found that duazomycin A was ineffective by itself, but it enhanced the suppressive activity of 6-mercaptopurine. Similarly, Elliott *et al.* (12) reported that medroxyprogesterone acetate was ineffective, but it potentiated the action of hydrocortisone acetate. Henson and Brunson (13) used a combination of epinephrine and propiomazine, but the effectiveness of the individual drugs was not evaluated. None of these reports have conclusive evidence for synergy.

The data of Table II readily satisfy the criteria of Sartorelli (9) and others for synergy. Although our experiments were not designed to meet the requirements emphasized by Berenbum (1), the data do satisfy his criteria:

$$(CL_s/CL_e) + (T_s/T_e) < 1,$$

where CL_e and T_e are the doses of cycloleucine given alone or tilorone given alone that are equi-effective with the dose CL_s combined with the dose T_s that is putatively synergistic. If the delays in Table II for CL, T, and $^1/_4CL+^1/_4T$ are accepted as sufficiently close to be considered equal, then the two fractions are each $^1/_4$, and their sum is much less than 1.

Despite the difficulty of these experiments, the extraordinary advantage obtained by some of the combinations in Table I warrants further investigation of optimum schedules and optimum doses, as well as the mechanism of the synergistic interaction. The mechanism may involve alterations of the pharmacokinetics of either or both drugs, or cooperative effects on the process of immunization. Progress in this area is hampered by incomplete understanding of drug mechanisms, but the possibility of clinical applications in autoimmune diseases justifies further studies.

Summary. Single doses of antilymphocyte serum, cyclophosphamide, cycloleucine, and tilorone delayed the onset of the hyperacute form of experimental allergic encephalomyelitis. Many combinations of drugs delayed the onset more than the sum of the delays attributed to each agent alone. The efficacy of some combinations depended on the schedule of administration. Cycloleucine and tilorone were proven to have a synergistic relationship.

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1. Berenbaum, M. C., Clin. Exp. Immunol. **28,** 1 (1977).
2. Levine, S., and Wenk, E. J., Amer. J. Pathol. **47,** 61 (1965).
3. Levine, S., and Wenk, E. J., Amer. J. Pathol. **50,** 465 (1967).
4. Paterson, P. Y., and Hanson, M. A., J. Immunol. **103,** 795 (1969).
5. Rosenthale, M. E., Datko, L. J., Kassarich, J., and Rosanoff, E. I., J. Pharmacol. Exp. Ther. **180,** 501 (1972).
6. Levine, S., and Sowinski, R., Exp. Mol. Pathol. **26,** 103 (1977).
7. Megel, H., Raychaudhuri, A., Goldstein, S., Kinsolving, C. R., Shemano, I., and Michael, J. G., Proc. Soc. Exp. Biol. Med. **145,** 513(1974).
8. Levine, S., *in* "Modulation of Host Immune Resistance in the Prevention or Treatment of Induced Neoplasia." (M. A. Chirigos, ed.), Fogarty Inter-

national Center Proceedings, No. 28. U.S. Government Printing Office, Washington, D.C. (1977).
9. Sartorelli, A. C., Prog. Exp. Tumor Res. **6,** 228 (1965).
10. Pellet, H., Brun, M., Camargo, R., and Girard, P., Rev. Neurol. **118,** 133 (1968).
11. Vogel, C. L., and Calabresi, P., Proc. Soc. Exp. Biol. Med. **131,** 251 (1969).
12. Elliott, G. A., Gibbons, A. J., and Greig, M. E., Acta Neuropathol. **23,** 95 (1973).
13. Henson, E. C., and Brunson, J. G., Proc. Soc. Exp. Biol. Med. **131,** 752 (1969).

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