REFERENCES

1. N. B. THOA and W. M. BOOKER, *Fedn Proc.* **22**, 448 (1963).
2. N. B. THOA, R. J. WURTMAN and J. AXELROD, *Proc. Soc. exp. Biol. Med.* **121**, 267 (1966).
3. B. BHAGAT, W. L. WEST and I. M. ROBINSON, *Biochem. Pharmac.* **15**, 1637 (1966).
4. B. B. BRODIE, E. COSTA, A. DLABAC, N. H. NEFF and H. G. SMOOKLER, *J. Pharmac. exp. Ther.* **154**, 3 (1966).
5. R. MONTARINI, E. COSTA, M. A. BEAVEN and B. B. BRODIE, *Life Sci.* **4**, 232 (1963).
6. L. L. IVERSEN and J. GLOWINSKY, *Nature, Lond.* **210**, 1006 (1966).
7. S. SPECTOR, A. SJOERDSMA and S. UDENFRIEND, *J. Pharmac. exp. Ther.* **147**, 86 (1965).
8. A. BERTLER, A. CARLSSON and E. ROSENGREN, *Acta physiol. scand.* **44**, 273 (1958).
9. T. BRAY, *Analyt. Biochem.* **1**, 279 (1960).
10. G. A. BRUNO and J. E. CHRISTIAN, *Analyt. Chem.* **33**, 650 (1961).
11. I. J. KOPIN, J. AXELROD and E. GORDON, *J. biol. Chem.* **236**, 2109 (1961).

Biochemical Pharmacology, Vol. 18, pp. 256–260. Pergamon Press. 1969. Printed in Great Britain

Inhibition of antibody synthesis by cycloleucine

(*Received* 16 *May* 1968; *accepted* 9 *August* 1968)

DURING the past decade, such immunosuppressive drugs as 6-mercaptopurine, thioguanine, methotrexate and cyclophosphamide have been identified and studied in detail.[1–5] Maximal inhibition of the immune response usually results when these and other agents are introduced within 18 to 48 hr after primary antigenic stimulus, i.e. during the induction phase. An exception to this general rule is the sulfonic acid esters, which mimic radiation effects and which are most inhibitory if administered prior to immunization.[2, 6, 7] The results of a study of another such immunosuppressant, the amino acid analogue cycloleucine (1-amino-cyclopentane-carboxylic acid), constitute the subject of this report. Pretreatment of Swiss mice with this drug prevents the synthesis of hemagglutinins and hemolysins for sheep erythrocytes by reducing the number of plaque-forming cells in the splenic pulp.

METHODS

The methodology, which has been described in detail elsewhere, consisted of administering the drug, suspended in methylcellulose, intraperitoneally and the immunization of mice with a single dose of washed sheep erythrocytes given via the same route. The animals were bled from the orbital sinus at selected intervals and the hemagglutinin titers determined.[4] Hemolytic titers were measured by adding an excess of guinea pig complement to the hemagglutination tubes and incubating for an additional hour at 37°. The number of plaques in the spleen was determined by the method of Jerne *et al.*[8] Statistical analysis of the titration data in the tables was carried out by using the t = test. In each instance the results were compared with those obtained from the control animals.

RESULTS

In the first three experiments maximal single doses of cycloleucine were given at various times in relation to the intraperitoneal injection of sheep erythrocytes. These data have been combined in Table 1. In part A, significant immunosuppression was achieved when cycloleucine was injected 3 days before, but not 3 days after, antigen. Additional experiments in parts B and C indicated that inhibition could still be obtained if pretreatment with cycloleucine was carried out up to 12 days prior to antigenic stimulation.

TABLE 1. INHIBITION OF HEMAGGLUTININ SYNTHESIS BY CYCLOLEUCINE

Cycloleucine (mg/kg)	Days before and after Antigen	No. of mice tested	Mean hemagglutinin titer (+10 days)
Part A			
500	−3	3	1·0*
	−1	4	1·0*
	0	6	1·3*
	+1	7	3·5*
	+3	7	6·2
400	−3	6	2·3*
	−1	7	1·2*
	0	6	1·0*
	+1	6	3·7*
	+3	7	6·0
None		7	7·0
Part B			
400	−6	7	1·0*
	−4	8	1·6*
	−2	8	1·0*
None		5	7·4
Part C			
350	−14	3	6·7
	−12	5	4·0*
	−10	6	5·2*
	−8	6	3·5*
	−6	6	2·5*
	−4	5	2·4*
None		5	8·4

*P value significant at the 5 per cent level or less when compared with control animals.

The titration data were confirmed by a similar experiment in which the Jerne plaquing technique was used.[8] The mice received 400 mg/kg of cycloleucine on the day indicated. A standard dose of 0·2 ml of packed sheep erythrocytes was given at zero time; the animals were tested 5 days after antigenic stimulus. These results, given in Table 2, represent duplicate tests from titrated pools of

TABLE 2. CYCLOLEUCINE BEFORE AND AFTER ANTIGEN: EFFECT ON ANTIBODY TITER AND ON PLAQUE COUNT IN THE SPLEEN*

Cycloleucine-antigen relationship (− or + in days)	No. of cells in the spleen ($\times 10^6$)	Plaques/ million spleen cells	Titer log base 2*	
			Hemagglut-ination	Hemolysin
−3	43·5	74·5	1·7†	1·0†
−2	42·8	16·4	1·0†	1·0†
−1	51·8	133·6	3·0†	1·0†
0	45·0	50·0	1·0†	1·0†
+1	40·0	73·9	3·0†	4·3†
+2	83·3	711·9	5·3	7·0
+3	111·3	688·0	3·7	6·7
Control	138·5	1,640·0	6·3	7·7

* Mean titers are based on 3 animals.

† Significant at the 5 per cent level or less when compared with control animals.

three mouse spleens. They show that cycloleucine inhibited plaque formation and hemagglutinin and hemolysin synthesis maximally when administered 3 days before and on the day antigen was injected. Only partial inhibition was obtained thereafter.

In the next experiment cycloleucine was given 2 days prior to antigen, but the dose was varied. The same mice were bled and titers were taken 7, 14, and 21 days after a single primary antigenic stimulus. These data are presented in Table 3. It may be seen that significant, but not complete, inhibition was obtained for the entire 21-day period with the minimal dose of 250 mg/kg.

TABLE 3. EFFECT OF VARYING DOSES OF CYCLOLEUCINE ON HEMAGGLUTININ TITERS

Cycloleucine dose (mg/kg)	7-day titers		14-day titers		21-day titers	
	No. mice tested	Log base 2	No. nice tested	Log base 2	No. mice tested	Log base 2
400	9	1·3*	5	3·6*	3	3·3*
350	8	1·9*	7	2·1*	4	1·5*
300	8	3·8*	8	3·6*	7	4·0*
250	6	4·7*	6	4·3*	6	4·3*
None	6	8·5	6	8·0	5	8·2

* Significant at the 0·5 per cent level or less when compared with the control animals.

In the next experiment a single dose of 400 mg/kg of cycloleucine was administered at −2 days, followed by 0·2 ml of packed sheep erythrocytes on day zero. A second dose of sheep cells was given to the drug-treated mice 2, 4 and 6 days after the first; titrations were carried out 7 days after the second stimulus for each group. As seen in Table 4, the mice given cycloleucine demonstrated a weak

TABLE 4. EFFECT OF TWO DOSES OF SHEEP ERYTHROCYTES ON HEMAGGLUTININ SYNTHESIS OF CYCLOLEUCINE-TREATED MICE

Day of second dose	Cycloleucine-treated groups		Untreated control groups	
	No. mice	Titer log base 2	No. mice	Titer log base 2
+2	9	2·3*	6	7·3
+4	9	4·3*	6	8·7
+6	5	4·2*	6	10·2

* Significant at the 0·5 per cent level or less when compared with control animals.

primary response and partial tolerance to the second stimulus. In the untreated mice, a secondary type response was induced by antigen given 6 days after primary immunization. Similar results were obtained in a separate experiment when the second antigen was given 10 days after the primary stimulus. Tolerance of the degree produced by cyclophosphamide was not obtained.[3, 9]

An effort was made to inhibit primary antibody synthesis with 5 repeated daily doses of cycloleucine (50 mg/kg). These effects were compared with an equal amount of drug (250 mg/kg) administered in a single dose. It can be seen from the data (Table 5) that the partial inhibition which occurred at both the serum and cellular level was neither augmented nor depressed by 50 mg/kg of cycloleucine given for 5 consecutive days. From these data the immunosuppression appears to be cumulative, since a single 50-mg dose is known to be ineffective. Attempts to reverse the effect by giving 1000 mg/kg of methionine before and after cycloleucine were not successful.

In the next experiment the immunosuppressive capacity of cycloleucine on the primary response was compared with six other cytotoxic agents administered 24 hr after antigenic stimulation. The

data, which are presented in Table 6, were obtained 5 days after antigenic stimulation and represent tests for 19S antibody; antimouse globulin to detect spleen cells demonstrating a 7S response[10] was not utilized.

TABLE 5. EFFECT OF REPEATED DOSES OF CYCLOLEUCINE ON PLAQUE, HEMAGGLUTININ AND HEMOLYSIN FORMATION

Cycloleucine daily dose	Plaques/million spleen cells	Titer log base 2	
		Hemagglutination	Hemolysin
50 mg/kg −4 thru 0	36·7	5*	5*
250 mg/kg −1	39·7	3·8*	4·0*
250 mg/kg	48·1	6·2*	6·2*
Control	683·5	8·5	10·0

* Significant at the 5 per cent level or less when compared with the control animals.

TABLE 6. COMPARISON OF CYCLOLEUCINE WITH OTHER IMMUNOSUPPRESSIVE DRUGS

Drug	Dose (mg/kg)	Nucleated cells/spleen ($\times 10^6$)	plaques/million spleen cells	Titer log base 2	
				Hemagglut-ination	Hemolysin sin
Cyclophosphamide	100	26·2	0	1*	1*
Triethylene-melamine	1·5	15·0	0	1*	1*
Uracil mustard	2·7	31·0	1·9	1*	1*
Thioguanine	20·0	64·0	2·0	1*	1*
Methotrexate	20·0	130·0	21·7	4·3*	6·6*
5–FUDR	350·0	123·0	55·6	5·0	7·6
Cycloleucine +24 hr	400·0	55·3	84·7	3·6*	5·0*
Cycloleucine −24 hr†	400·0	68·0	20·3	1·7*	2·0*
Control		131·5	285·7	7·3	9·2

* Significant at the 5 per cent level or less, when compared with control animals; 800 mg/kg of 5–FUDR and 75 mg/kg of methotrexate showed more pronounced hemagglutinin inhibition than that given in the table.

† All other drugs given 24 hr after antigenic stimulation.

The most effective immunosuppressants at the dose levels and times employed were cyclophosphamide, triethylenemelamine, uracil mustard and thioguanine. The remainder were inhibitory but less active as measured by the three criteria employed, i.e. hemagglutination, hemolysis and 19S plaque formation. The previous observation on the effectiveness of pretreatment with cycloleucine was confirmed. The relative cytotoxicity of the drugs for lymphoid tissues can be estimated by the depression of total spleen cell counts as shown in Table 6.

The effect of cycloleucine on the secondary response was tested in the following experiment. Initially the mice received a primary intraperitoneal stimulus with 0·4 ml of 50% sheep erythrocytes. A second injection of erythrocytes was given 14 days later at zero time. A dose of 400 mg/kg of cycloleucine was administered before and after the secondary stimulus on the days shown in Table 7. The animals were bled from the orbital sinus 8 days later and serum was obtained. Jerne plaque procedures for both 19S and 7S type antibody were carried out by using a pool of three spleens for each assay.[10] Although cycloleucine produced a distinct reduction in the number of nucleated cells in the spleen, there was no significant inhibition of 19S or 7S plaque formation. The titration data also indicate that cycloleucine does not affect the secondary hemagglutinin and hemolysin response.

TABLE 7. EFFECT OF CYCLOLEUCINE ON THE SECONDARY RESPONSE

Cycloleucine (400 mg/kg) on Day	Nucleated cells/spleen ($\times 10^6$)	Plaques/10^6 spleen cells		Mean titer, log base 2		
		19S Direct	19S+7S Antiglobulin	Hemagglutination	Hemolysin	No. of mice
−4	9·6	75	689	9·3	9·7	7
−2	8·3	65	100	9·7	10·0	8
0	10·1	346	1090	9·1	10·0	8
+2	9·0	164	312	9·1	9·9	8
None	21·8	79	222	10·3	11·0	8

Since a number of cycloaliphatic amino acids have been described,[11] a few such compounds were obtained and tested.* They included the carboxylic acid derivatives of the following: 1-aminocyclopropane, 2000 mg/kg; 1-aminocyclobutane, 2000 mg/kg; 1-aminocyclohexane, 4000 mg/kg; 1-aminoindane, 2000 mg/kg; 1-aminotetrahydronaphthalene, 2000 mg/kg; and in addition, cycloserine, 0·5 mg/kg. All of them lacked immuno-suppressive activity at the dose levels indicated.

DISCUSSION

The cytotoxicity of cycloleucine for experimental animals and man has been reported.[11, 12] Biochemical studies have revealed that it does not act as a metabolic analogue,[13] but that it does share a common transport system with DL-methionine.[14] As an immunosuppressant, cycloleucine acts on the preinduction phase, in contrast to thioguanine and cyclophosphamide, which interfere with the induction phase of humoral antibody synthesis.[3, 4 9] The inability of cycloleucine to inhibit the secondary antibody response also marks it as a less potent immuno-suppressive drug. In a limited trial with inbred strains of mice differing at the H_2 locus, we did not obtain increased graft survival with cyclcoleucine. Perhaps a less toxic analogue can be synthesized in the future.

Department of Microbiology,
University of Oregon Medical School,
Portland, Oregon, U.S.A.

ARTHUR W. FRISCH

*We are greatly indebted to the Chester Beatty Research Institute, Royal Cancer Hospital, Fulham Road, London, S.W.3, England, for providing these compounds.

REFERENCES

1. A. C. AISENBERG, *J. exp. Med.* **125**, 833 (1967).
2. M. C. BERENBAUM, in *Symposia on Immunity, Cancer and Chemotherapy*, Immunosuppressive agents and the cellular kinetics of the immune response (Ed. E. MIHICH) Acadamic Press, Buffalo, New York (1967).
3. A. W. Frisch and G. H. Davies, *J. Lab. clin. Med.* **68**, 103 (1966).
4. A. W. FRISCH, G. H. DAVIES and V. MILSTEIN, *J. Immun.* **88**, 269 (1962).
5. R. S. SCHWARTZ, *Prog. Allergy* **9**, 246 (1965).
6. M. C. BERENBAUM, *Biochem. Pharmac.* **2**, 29 (1962).
7. M. C. BERENBAUM, G. M. TIMMIS and I. N. BROM, *Immunology* **13**, 517 (1967).
8. N. K. JERNE, A. A. NORDIN and C. HENRY, Cell-bound Antibodies, p. 109, *Wistar Institute Press*, Philadelphia (1963).
9. A. W. FRISCH and G. H. DAVIES, *Cancer Res.* **25**, 745 (1965).
10. D. W. DRESSER and H. H. WORTIS, *Nature, Lond.* **208**, 859 (1965).
11. R. B. ROSS, C. I. NOLL, W. C. J. ROSS, M. V. NADKARNI, B. H. MORRISON, JR., and H. W. BOND, *J. Mednl pharm. Chem.* **3**, 1 (1961).
12. W. H. TALLIAFERRO, *Ann. N.Y. Acad. Sci.* **69**, 745 (1957).
13. M. N. MICKELSON and R. S. FLIPPIN, *Progress Report No.* 34. Midwest Research Inst., Kansas City, Kansas. (Cited on page 16, ref. 11).
14. K. AHMED and P. G. SCHOLEFIELD, *Can. J. Biochem. Physiol.* **40**, 1101 (1962).