liquid nitrogen at the following temperatures during freezing: 0, −10, −20, −30, −40, −50, −60, −70, −79, −100, −120 and −196 °C. For thawing, samples were put in air at room temperature and then embryos were washed with culture medium. Embryos were cultured by the microdrop method in a modified Krebs-Ringer bicarbonate medium[5] at 37 °C for 36 h in 5% CO_2 in air. Since development of frozen-thawed mouse embryos in vitro was highly correlated with the normal embryonic development in vivo after transfer[6], survival was assessed by the ability of frozen-thawed morulae to develop into expanded blastocysts during culture. Experiments were replicated 4–5 times and statistical significance was determined by the χ^2 test.

Results and discussion. The results are summarized in the table. When embryos were cooled in the presence of DMSO, almost no decrease in survival occurred between 0 and −50 °C and a small decrease ($p < 0.05$) was observed after exposure to −60 °C. Compared with survival of embryos exposed to 0 °C, a large decrease ($p < 0.001$) in survival occurred after exposure to −120 and −196 °C. After cooling embryos in the presence of glycerol, almost no damage to embryos occurred at 0 to −40 °C. Considerable decrease ($p < 0.001$) in survival, however, occurred at −50 °C or lower temperatures, and especially a large damage ($p < 0.001$) was observed after exposure to −120 and −196 °C. When embryos were cooled in the presence of ethylene glycol, survival hardly decreased between 0 and −40 °C. Compared with survival of embryos exposed to 0 °C, a small decrease ($p < 0.05$) in survival was observed after exposure to −50 °C and no more damage occurred after exposure to temperatures below −50 °C in the presence of ethylene glycol.

These results show that when mouse morulae are cooled to −79 °C at 0.5 °C/min before cooling to −196 °C in the presence of DMSO, glycerol or ethylene glycol, damage to embryos occurs after exposure to −60, −50 or −50 °C, respectively and also show that particularly when DMSO or glycerol was used as the cryoprotectant, survival of morulae are dependent upon the low temperature to which they are exposed.

When unfertilized mouse ova in DMSO were cooled to −30, −45 and −75 °C, survival was 51, 56 and 18%, respectively[4]. Following the transfer of rat embryos in DMSO unfrozen or frozen to −50 and −79 °C into recipient rats, 100, 83 and 64% of recipients became pregnant, respectively[7]. After sheep embryos in DMSO were cooled to −25, −45 and −60 °C, survival was 100, 67 and 75%, respectively[8]. Results in the present study are similar to the earlier reports regarding unfertilized mouse ova[4], rat[7], sheep[8] embryos.

A major cause of freezing injury is intracellular ice formation[2] and intracellular freezing of unfertilized mouse ova in 1 M DMSO occurs at about −40 to −45 °C[3]. The present results may support the claim[3] that mouse embryos still contain water capable of freezing intracellularly at temperatures above −50 °C.

When mouse morulae were frozen to −196 °C in the presence of DMSO, glycerol and ethylene glycol, survival rate was 67, 43 and 88%, respectively. Ethylene glycol, therefore, afforded more ($p < 0.01$) effective protection to mouse morulae frozen to −196 °C than DMSO or glycerol, and DMSO better ($p < 0.01$) than glycerol. The present experiments also confirmed the report[5] that greater survival is obtained for mouse embryos frozen in the presence of DMSO than in glycerol.

1 R.R. Maurer, H. Bank and R.E. Staples, Biol. Reprod. *16,* 139 (1977).
2 P. Mazur, in: Cryobiology, p. 213. Ed. H.T. Meryman. Academic Press, New York and London 1966.
3 S.P. Leibo, J.J. McGrath and E.G. Cravalho, Cryobiology *15,* 257 (1978).
4 T.A. Parkening, Y. Tsunoda and M.C. Chang, J. exp. Zool. *197,* 369 (1976).
5 D.G. Whittingham, S.P. Leibo and P. Mazur, Science *178,* 411 (1972).
6 D.G. Whittingham, Cryobiology *15,* 245 (1978).
7 K. Utsumi and M. Yuhara, Sci. Rep. Fac. Agr. Okayama Univ. *44,* 24 (1974).
8 S.M. Willadsen, C. Polge, L.E.A. Rowson and R.M. Moor, J. Reprod. Fert. *46,* 151 (1976).

Cyclosporins: Immunosuppressive agents with antitumor activity[1]

W. Kreis and Angela Soricelli[2]

Memorial Sloan-Kettering Cancer Center, New York (New York 10021, USA), 6 February 1979

Summary. Initial screening of the 2 recently developed immunosuppressive agents, cyclosporin A and cyclosporin C, in 11 murine transplantable neoplasms revealed significant increase of lifespan with long-term survivors after i.p. injection to the ascites tumors, Taper liver, Sarcoma 180J and Ehrlich.

The biological activity of cyclosporins, a group of antifungal metabolites recently isolated from *Trichoderma polysporum* (Link ex Pers.) Rifai[3–7], consists in the inhibition of humoral immunity as evidenced by the reduction of plaque-forming cells and haemagglutinin titers in mice[8]. Suppression of cell-mediated immunity was demonstrated in test systems for skin graft rejection, graft-versus-host disease and experimental allergic encephalomyelitis in rodents[8]. The most studied representative is cyclosporin A, a neutral cycloendecapeptide with several N-methylated amino acids and 1 new amino acid. This new agent proved to be very active in chronic inflammatory reactions and produced only mild myelotoxicity[8]. It displayed inhibition of transplant rejections in patients receiving renal allografts[9] and graft-versus-host disease in man[10]. The drug delayed the hypersensitivity skin reaction to oxazolone in mice and to tuberculin in guinea-pigs. It did not suppress antibody synthesis to lipopolysaccharide antigens in nude mice, indicating a selective effect upon T-cells[11]. Further evidence for selectivity for T-cells was also provided in in vitro lymphocyte proliferation tests[12]. The present exploratory study was undertaken to test for possible antitumor activity of 2 representatives of this new group of compounds.

A series of solid and ascitic mouse tumors [mast cell tumor, ascites (P815), leukemia L1210 (L1210), Taper liver tumor, ascites (Taper), Sarcoma T241, solid (T241), adenocarcinoma EO771, solid (EO771), Sarcoma 180J, ascites and solid (S180J), T-cell tumor EL-4, ascites and intradermal (EL-4), leukemia L5178Y resistant to 1-β-D-arabinofuranosylcytosine, ascites (5178Y/CA-55), Ehrlich ascites (Ehrlich), Ridgeway osteogenic Sarcoma (ROS), Lewis lung carcino-

Effect of i.p. administered CyA and CyC to murine neoplasms in ascites form

Neoplasm treatment	P 815			L 1210			L 1210		Ehrlich			Taper	Liver	S 180 J		
Cyclosporin A																
Vehicle	A[a]	A	A	A	A	A	A	A	A	A	A	A	A	A	B[b]	B
Dosage (mg/kg)	75	85	95+75	75	85	95	85	95	75	85	95	75	85	75	75	85
Number of injection[c]	7	6	2+2	6	6	4	7½[d]	7½	7	7	4	7	7	7	7	7
T/C[e]	1.4	1.6	1.0	1.4	1.6	1.0	1.3	1.2	1.2	1.0	0.3	1.3	1.6	2.8	1.7	1.4
30-day survivors	0	0	0	0	0	0	0	0	0	0	1	0	2	3	0	1
ΔWt day 6 (g/mouse)	+1.0	−0.4	−3.0	+3.6	0	−2.0	+2.6	+3.6	−1.2	−2.4	−2.0	−1.2	−0.8	−2.8	−2.6	−1.6
Remarks			Toxic			Toxic					Toxic	Toxic				Toxic Toxic
Cyclosporin C																
Vehicle	A	A	A	A	A		A	A	A	A	A	A	A	A	B	B
Dosage (mg/kg)	75	85	95+75	75	85		85	100	75	85	95	75	85	75[f]	75	85
Number of injection	7	6	2+2	6	6		7½	7½	7	4	7	7	7	7	7	7
T/C	1.8	1.8	0.8	1.4	1.6		1.3	1.5	1.6	0.8	1.2	1.9	1.8	>2.8	1.0	1.8
30-day survivors	0	0	0	0	0		0	0	2	1	0	4	3	5	0	2
ΔWt day 6 (g/mouse)	−1.0	0	−1.6	−0.8	−3.8		+2.6	+0.4	−1.6	−1.2	−2.4	−2.2	−1.6	−2.0	−2.0	−2.0
Remarks			Toxic		Toxic						Toxic					
Controls																
Vehicle		A			A		A	A		A			A	A[f]	B	
MST[g] (days)		5			5		6	6		17			19	12	12	
30-day survivors		0			0		0	0		1			0	0	0	
ΔWt day 6		+5.0			+5.2		+4.2	+4.8		+2.6			+1.0	+2.2	+1.8	

[a] Vehicle A consists of 5 ml of 95% ethanol, 5 ml of Tween 80 and 40 ml of saline. [b] Vehicle B consists of 5% carboxymethylcellulose (CMC). [c] Administration of drug on consecutive days. [d] One-half of dosage administered in the morning; one-half in the afternoon. [e] T/C: Treated over Control: The median half-life of the control vs. treated animals was used for the evaluation of the therapeutic effect. [f] Vehicle A, when injected in amounts larger than 0.4 ml/20 g mouse can significantly prolong the survival time of S180J-bearing mice. Care was taken that the amount injected into S180J-bearing mice was below 0.34 ml/20 g mouse and produced the same median survival time of the control animals as did saline or 5% CMC. Therefore, the relatively low solubility of both cyclosporins did not allow a higher dosage than 75 mg/kg. None of the other neoplasms tested showed such a sensitivity to Vehicle A. [g] MST: Median survival time.

ma (LL)], presently maintained by serial transplantation or stored in liquid nitrogen at this Institute, were implanted s.c. by trocar or by i.p. or i.d. (intradermal) injection of about 10^6 cells, in appropriate strains of mice to groups of 5 mice. Except for ROS, which was grown in $ADK2F_1$ mice, female BDF_1 mice 18–20 g were the tumor hosts. Treatment was started 24 h after the inoculation of the tumor and continued daily for 7 days (or as indicated in the table). The evaluation of the results was done by measurement on days 8 and 15 after inoculation by adding the longest and the shortest tumor diameters or by evaluating the survival time of the ascites-bearing mice. Toxicity of the substances was defined by early death, treatment vs. control T/C values substantially below 1.0 or weight loss in excess of 2.5 g/mouse on day 5 or 6 after inoculation. Cyclosporin A (CyA)[7] and its congener, cyclosporin C (CyC)[7], were dissolved in 95% ethanol at a concentration of 50 mg/ml to which Tween 80 (Fisher Scientific Co., Fair Lawn, NJ) was added at a volume equal to the one of alcohol, and this clear solution diluted with physiological saline so that in the final solution the alcohol concentration did not exceed 10%. In some instances a suspension of the material was prepared by homogenizing CyA or CyC in 0.5% carboxymethylcellulose (CMC). The control animals were injected with the respective vehicle alone. The dosage in all experiments described was optimal or close to optimal and was in most instances close to the tolerable toxicity.

With the excpetion of S180J, Ehrlich and Taper liver, the tumors tested are considered to be syngeneic and consequently of low immunogenicity. Sarcoma S180J, Ehrlich and Taper liver arose originally in noninbred mice and thus there are no syngeneic mice for these tumors. One would expect these tumors to exhibit medium to high immunogenicity in a variety of inbred hosts. The S180J line used in this study was obtained in the ascites form in 1973 from Dr Chihara of the National Cancer Research Institute, Tokyo, Japan. This Japanese line of S180, derived from the Sloan-Kettering Institute line, was selected for use because of its low rate of spontaneous regression in strains derived from ICR/Ha mice[13].

Of the 6 solid tumors tested (T241, EO771, LL, ROS, S180J and EL-4) none responded to 50–100 mg/kg of the 2 drugs. In all tumors tested, 75 mg/kg produced slight-to-lethal toxicity in the form of severe weight loss or early death. Weight loss was most significant in the case of LL and ROS. It is of particular interest that S180J when inoculated s.c. did not respond at all to the treatment with the 2 cyclosporins, whereas the ascitic form exhibited excellent response to both compounds, as evidenced by increased T/C and 30-day survivors (table). In contrast, the T-cell tumor EL-4 showed no significant response in either ascites or solid form (i.d.).

Of the 7 ascites tumors tested (P815, L1210, Ehrlich, Taper, EL-4, L5178Y/CA-55 and S180J), only L5178Y/CA-55 showed no response at all, and the T-cell tumor EL-4 showed only marignal response to 75 mg/kg of both compounds. As presented in the table, P815 and L1210 exhibited significant T/C values but without producing 30-day survivors. Ehrlich ascites responded well to 75 mg/kg of CyC with 2 30-day survivors, but was toxic at 85 mg/kg. The most significant response of all these neoplasms was observed in Taper liver and S180 tumors. In both cases the T/C were elevated to almost double and triple, respectively, compared to the values of the controls, and 4 and 5 30-day survivors, respectively, were observed. However, some toxicity was observed in the S180J-bearing group receiving 75 mg/kg of CyA per day for 7 days.

It is of particular interest that definite inhibition of tumor growth occurred only with the ascites tumor and only with those tumors which originated in random-bred mice (S180J, Ehrlich, Taper liver). Admittedly, administration of drug i.p. in the case of the ascites tumors resembles in vitro

treatment of tumor cells. However, an indication of potential antitumor activity of a drug can thus be demonstrated, especially if the activity is weak. The systemic antitumor activity of the drugs would be revealed by inhibition of growth of solid tumors, especially when treatment is initiated early, 24 h after tumor implantation. The antigenicity of the tumors toward the hosts used are not known; to determine these for the exploratory work carried out here would not be feasible. Except for our inexplicable 30-day survivor (this might be due to accidental s.c. rather than i.p. inoculation since a solid tumor but no ascites was observed in this control mouse at autopsy on day 30) in the case of Ehrlich ascites, not seen before in our laboratories[14], the tumors in control mice grew progressively to kill the host. Such continuous growth suggest a low antigenicity for these tumors in the present situation. In addition, the reported suppressive activity of cell-mediated immunity by the cyclosporins should favor tumor growth rather than tumor regression. Therefore, the antitumor activity seen appears real. Discrepancies in the response of solid versus ascites tumors to the same agents as observed in these experiments are not new and have been reported earlier for other antitumor agents[15,16]. Resorption, distribution and rapid metabolism may be responsible for this phenomenon.

In those experimental test systems which responded to treatment, the comparison of the 2 drugs reveals similar efficacy; although in some, but not all instances, CyC was somewhat more effective. In S180J the use of 0.5% carboxymethyl cellulose resulted in substantial loss of therapeutic effect, while toxicity in the form of weight loss was comparable to the ethanol-Tween 80-saline group.

In L1210 the use of divided doses of CyA and CyC (table) (one-half each of the dosage administered in the morning and afternoon, respectively) did not improve the antitumor effect but was less toxic to the host. The results obtained in this initial screening program are encouraging and justify further work with this new group of compounds.

1 This work was supported in part by grant CA-08748 from the National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare.
2 The authors are indebted to Drs Dorris J. Hutchison, M.N. Teller and H. Stähelin for helpful discussions, and to Dr A. von Wartburg of Sandoz AG, Basel, for the supply of the cyclosporins.
3 J.F. Borel, A. Rüegger and H. Stähelin, Experientia *32*, 777 (1976).
4 A. Rüegger and A. von Wartburg, Helv. chim. Acta *59*, 1075 (1976).
5 R. Traber, M. Kuhn, A. Rüegger, H. Lichti, H-R. Loosli and A. von Wartburg, Helv. chim. Acta *60*, 1247 (1977).
6 R. Traber, M. Kuhn, H-R. Loosli, W. Pache and A. von Wartburg, Helv. chim. Acta *60*, 1568 (1977).
7 M. Dreyfuss, E. Härri, H. Hofmann, H. Kovel, W. Pache and H. Tscherter. Eur. J. appl. Microbiol. *3*, 125 (1976).
8 T.F. Borel, C. Feurer, H.U. Gubler and H. Stähelin, Agents Actions *6*, 468 (1976).
9 R.Y. Calne, S. Thiru, P. McMaster, G.N. Craddock, D.J.G. White, D.B. Evans, D.C. Dunn, B.D. Pentlow and K. Rolles, Lancet *2*, 1323 (1978).
10 R.L. Powles, H. Clink, J. Sloane, A.J. Barrett, H.E.M. Kay and T.J. McElwain, Lancet *2*, 1327 (1978).
11 J.F. Borel, C. Feurer, C. Maguée and H. Stähelin, Immunology *22*, 1017 (1977).
12 J.F. Borel and D. Wiesinger, Proc. 11th Leukocyte Culture Conf., p.716. Academic Press, New York, San Francisco, London 1977.
13 J. Chihara, J. Hamuro, Y.Y. Maeda, Y. Arai and F. Fukuoka, Cancer Res. *30*, 2776 (1970).
14 G.S. Tarnowski, I.M. Mountain and C.C. Stock, Cancer Res. *33*, 1885 (1973).
15 W. Kreis, N.Y. Acad. Sci. *255*, 244 (1975).
16 I. Wodinsky, P.C. Merker and J.M. Venditti, J. nat. Cancer Inst. *59*, 405 (1977).

The effects of decreased glucose concentrations on the in vitro development of the post-blastocyst mouse embryo in a fetal calf serum- or bovine serum albumin-supplemented medium

J.T. Hendryx[1] and R.J. Wordinger[1]

St. Bonaventure University, Department of Biology, St. Bonaventure (New York 14778, USA), 12 February 1979

Summary. Decreasing the glucose concentration from 1.0 mg/ml to 0.25 mg/ml has no detrimental effects on post-blastocyst embryo development when either dialyzed fetal calf serum (20%) or bovine serum albumin (4.0 mg/ml) is used to supplement Eagle's Basal Medium (BME). Development is reduced in both serum- and BSA-supplemented BME devoid of glucose in comparison to glucose controls. Serum-supplemented media support better overall development than BSA-supplemented media.

Successful in vitro culture of post-blastocyst mouse embryos to the early somite stage has been accomplished[2,3]. Numerous factors influence the successful culture of these embryos and opinions differ as to which medium is optimal for studying post-blastocyst embryo development[4-6]. The specific growth factors contained within the milieu of serum that usually supplements a particular chemically defined medium are only slowly becoming apparent[7,8]. In our laboratory, fetal calf serum-supplemented Eagle's Basal Medium (BME) has proven optimal for blastocyst culture[9]. Supplementation of BME with crystalline bovine serum albumin (BSA) has also been useful in some studies[7]. Information regarding carbohydrate requirements and metabolism during the period of post-blastocyst development has been limited. In vitro hatching, attachment, and outgrowth of mouse embryos are influenced by media glucose concentrations[10,11]. The objective of the present study is to observe the effects of reduced glucose levels on the in vitro development of the post-blastocyst mouse embryo in dialyzed fetal calf serum- and bovine serum albumin-supplemented BME.

Mouse blastocysts were cultured in either dialyzed fetal calf serum- or BSA-supplemented BME containing various concentrations of glucose. BME devoid of a carbohydrate energy substrate was prepared from commercially available amino acid, vitamin, and phenol red concentrates, and a laboratory-prepared Earle's balanced salt solution[12] supplemented with antibiotics. Prior to the addition of glucose, 1 volume of BME was supplemented with 20% dialyzed fetal calf serum which had been previously heat-inactivated at 56 °C for 0.5 h. Dialyzed fetal calf serum (dFCS) was chosen as a medium supplement in order to minimize the presence of small carbon moieties[10]. Analysis of both the BME and dFCS indicated no detectable levels of glucose. A 2nd volume of BME was supplemented with 4.0 mg/ml BSA. BSA was chosen as a supplement to maintain an