only component of cycad meal which increases liver cholesterol.

The marked similarity in effects of cycad meal and cycasin on RNA and phospholipid concentrations, which also correlate closely with the histologically observed disintegration of the cytoplasmic basophilia and of the loss of ribosomes from the parallel arrays of the endoplasmic reticulum, is evidence that a primary effect of the cycad toxins in liver cells may be localized at the endoplasmic reticulum. Whether the main effect is on the lipoprotein-rich membrane of the endoplasmic reticulum, whose destruction would cause a loss of positions for the attachment of the ribosomes, or whether the effect is on RNA synthetic pathways or directly on RNA by alkylation of the bases is not clear. This relationship is being studied.

Summary. The feeding of ground *Cycas circinalis* L. endosperm or cycasin, a glycoside isolated from the cycad endosperm, both of which produce liver and kidney tumors in rats and liver tumors in guinea pigs, produces an almost identical loss of RNA and phospholipids from rat liver cells. The ground endosperm is considerably more active, based on its cycasin content, than is cycasin. These changes correlate closely with loss of cytoplasmic basophilia, as observed with the light microscope, and with loss of ribosomes from the parallel arrays of the lipoprotein-rich endoplasmic reticular membrane, as observed with the electron microscope. No effect on DNA concentration occurred. Liver cholesterol and neutral glycerides were increased by cycasin feeding.

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1. Whiting, M. G., Economic Botany, 1963, v17, 270.

2. Laqueur, G. L., Mickelsen, O., Whiting, M. G., Kurland, L. T., J. Nat. Cancer Inst., 1963, v31, 919.

3. Magee, P. N., Farber, E., Biochem. J., 1962, v83, 114.

4. Schneider, W. C., in Methods in Enzymology, 1957, vIII, 680.

5. Davidson, J. N., Waymouth, C., Biochem. J., 1944, v38, 379.

6. Fisher, C. H., Subbarow, Y., J. Biol. Chem., 1925, v66, 375.

7. Bragdon, J. H., ibid., 1951, v190, 513.

8. Williams, J. N., Jr., Anderson, C. E., Jasik, A. D., J. Lipid Research, 1962, v3, 378.

9. Pearson, S., Stern, S., McGavack, T. H., Anal. Chem., 1953, v25, 813.

10. Folch, J., Lees, M., Sloane-Stanley, G. H., J. Biol. Chem., 1957, v226, 497.

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Specific Complement-Fixing Tumor Antigen in SV_{40}-Transformed Human Cells.* (29741)

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The presence of a specific complement-fixing (CF) antigen in SV_{40}-induced tumors and in SV_{40}-transformed cells was demonstrated by Black *et al*(1) through the use of serum antibodies produced in SV_{40} tumor-bearing hamsters. The same antigen was shown to be present not only in hamster tumors but in hamster, rabbit, mouse, pig and bovine cells "transformed" by SV_{40} virus *in vitro*, and their experimental findings suggested that this CF antigen was not identical to structural antigens of the virus. However, its specificity indicated that the information

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TABLE I. Presence of CF Antigen in SV_{40}-Transformed Human Cell Lines.

Antigen (10%)	Exposure to SV_{40} virus	Virus release	PPLO	Transformed	CF titer
Hamster SV_{40} tumor	+	—	?	+	32
WI-38	—	—	—	—	<4
W-18 CM	—	—	—	—	<4
HeLa	—	—	—	+	<4
KB	—	—	—	+	<4
WI-38 Va_1	+	+	+	+	32
WI-38 Va_2	+	+	+	+	64
W-18 Va_2	+	—	—	+	512
W-34 Va_1	+	+	—	+	128
W-89 Va_1	+	+	—	+	128
W-5 Va_2	+	+	+	+	64
WI-26 Va_4	+	—	—	+	32

for the CF antigen might come from viral genome in the transformed cells.

Using the same serological test system we have demonstrated the same specific CF activity in human cells transformed *in vitro* by SV_{40} and shown that this character is stable after transplant passage through human volunteers. Sera of these volunteers obtained before and after transplantation were tested for the presence of CF antibodies against hamster SV_{40} tumor antigen with negative results, although several of the human sera had demonstrable CF antibodies against an antigen present in normal hamster tissues.

Materials and methods. Antigens. Tumors or cells harvested from tissue culture were made up to 10-20% suspensions in veronal buffer, kept frozen at —50°C and used as such. For titrations of antibody, standard suspensions of a transplantable SV_{40} hamster tumor(2) were used at dilutions containing 4-8 units of CF antigen.

Antisera. All sera were inactivated at 60°C for 20 minutes. For titration of antigens a single positive hamster serum pool was used at a 1/32 dilution representing 4-8 units of CF antibody. This serum was negative when tested against hamster tumors produced by polyoma, adeno 12 and adeno 18 viruses, and SV_{40} virus antigen.

CF test. Standard Bengston procedure using 0.2 ml amounts of each ingredient was used with 2 full units of guinea pig complement and fixation overnight at 4°C. All endpoints are expressed as the reciprocal of that dilution giving 3+ fixation. In all tests standard antigen or antiserum was titrated as a positive control for sensitivity.

Transplantation in human volunteers. These procedures have already been described(3). In general, 5×10^6 tissue culture cells were inoculated intracutaneously into volunteer patients suffering from incurable cancer. The inoculated area was observed for nodule development and the nodule surgically removed at 7 days. The biopsied nodule in some instances was reestablished in tissue culture.

Cell lines. These have been described in previous publications(4,5). Criteria of *in vitro* "transformation" were changes in morphology, lack of mitotic inhibition and karyotypic abnormalities.

Test for presence of infectious virus. Green monkey kidney cultures were exposed to the following preparations of infected human cultures: 1) Fluid medium, 2) Cell extracts obtained by repeated freezing and thawing and 3) Cell extracts obtained by sonication. In addition, cells obtained from human cultures infected with SV_{40} were grown on the feeder layer of green monkey kidney cells which were subcultured twice and observed for lesions for a period of 6 weeks(6).

Results. Presence of CF antigen in human cells transformed by SV_{40}. In Table I are given the results of antigen titrations on the SV_{40} hamster tumor and various human cell cultures. All those containing "Va" in their designation had been exposed to SV_{40} and now showed the characteristics of transformed cells. Detection of CF antigen was not correlated with the presence or absence of virus or PPLO, but was consistently found in those cells transformed by SV_{40}. Titers of 10% cell suspensions varied from 1/32 to 1/512

TABLE II. CF Antigen Titers of SV_{40}-Transformed Human Cells After Transplant Passage in Man.

Antigen (10%)	No. of transplant passages in man	Virus release	CF titer
W 537	1	—	>32
W 529	1	—	16
W 561	1	—	>32
W 580	2	—	>64
W 588	2	—	>64
W 580 E	2	—	>32

but in general were consistently higher than titers usually found in our transplantable hamster tumors. Normal human diploid cells never exposed to SV_{40} and both neoplastic HeLa and KB cultures which originated from human tumors were negative for antigen. Several of the positive transformed human cell lines were tested and found negative against a standard positive adenovirus 12 anti-tumor hamster serum.

Persistence of CF antigen after transplant passage in man. As a part of other studies concerning the ability of transformed human cells to multiply in man, a number of cancer patient volunteers were inoculated intracutaneously with 5×10^6 W-18 Va_2 or other similar cells(4). In many of these patients a small localized nodule developed over a period of 1 week, at which time it was usually surgically removed. In some cases this biopsy tissue was trypsinized, planted in tissue culture, grown until confluent monolayers had developed, at which time the cells were harvested, tested for the presence of the CF antigen and implanted again in man(7). Table II shows that cell cultures from the developing nodules of 3 primary implants of W-18 Va_2 cells and 3 after their second transplant passage(7) in man were all positive for the specific antigen. Nodules were never produced by normal uninfected diploid cells.

CF antibody titers in patients inoculated with SV_{40} transformed human diploid cells. Serum specimens obtained before and after patient volunteers were inoculated with SV_{40}-transformed human cells were tested for CF antibodies against hamster SV_{40} tumor antigen(7). Table III lists the patients by number, the type of tissue culture cells inoculated, and the CF antibody titers of sera obtained before and after the cell transplant. Of a total of 18 different patients, 5 had post-transplant sera so highly anticomplementary that the results could not be evaluated and this was not changed by adsorption with kaolin. Of the 13 patients on whom satisfactory sera were available, 6 had titers of greater than 1/4. However, 5 of these 6 patients had equally as high titers in their sera obtained before inoculation of the transplant, and moreover these sera also reacted with polyoma and adeno 18 virus hamster tumor antigens and with normal adult hamster heart and kidney antigens. The reaction pattern of the sera was not affected by adsorption with kaolin. Furthermore, 4 of the positive sera did not react in the complement-fixation test when extracts of W-18 Va_2 human transformed cells or WI-38 normal human diploid cells were used instead of hamster tumor antigen. Pre- and post-transplant sera from patient W 536 which had shown a rise in titer from less than 1/4 to 1/8 against SV_{40} tumor antigen of hamster origin were both negative against extracts of W-18 Va_2 human SV_{40}-transformed cells. Reaction patterns in 4 patients who received implants of virus-shedding cells (Table III) did not differ in any way from those who received virus-free cells. Finally, there was no evidence of a specific CF antibody response in the 4 patients who received 2 separate successive transplants of SV_{40}-transformed cells, nor did they show any resistance to nodule formation from the second transplant inoculation(7).

Discussion. It has clearly been demonstrated that human cells transformed by SV_{40} *in vitro* as evidenced by morphology, growth pattern and karyotype contain the CF antigen previously found in SV_{40}-induced hamster tumors and in other species of cells transformed by this virus *in vitro*. Although Shein *et al*(8) mention the presence of such type of antigen in one "exceptional transformed human cell line" which still contained infectious SV_{40}, the antigen demonstrated in our experiments was extracted from cells unrelated to the presence or absence of infectious virus (Table II). The demonstrated persistence without loss of titer

of the specific CF antigen in these human transformed cells after one and two transplant passages in man illustrates the utility of this antigen as a cell marker in situations where transformed cells are being placed into mixtures with other cells and subsequent specific identification is important.

The results of the CF tests with sera from patients supporting the growth of nodules from transplants of SV_{40}-transformed human cells reveal 2 points of possible interest in the area of tumor-virus immunology. There was a lack of any tumor specific antibody response to the growth of the transplant cells containing an abnormal antigen. This may have been due to the fact that following homologous implantation of transformed human cell lines the number of potential

TABLE III. CF Antibody Titers in Sera of Cancer Patient Volunteers Before and After Transplant Inoculation of SV_{40}-Transformed Human Cell Cultures.

		Transplant inoculated			
Patient	Cell line	State of cells†	Date	Date bled	CF titers‡
W 5	W-5 Va_1§	T	2/27/62	2/ 6/62	4
	W-5§	N	6/11/62	6/25/62	4
	W-5 Va_2§	T	5/ 9/63	5/ 9/63	4
				7/22/63	4
W 29	WI-26 Va§	T	4/16/62	4/16/62	<4
				6/27/62	<4
W 501	W-18 Va_2	T	1/31/63	1/31/63	>64
	HeLa	T*	1/31/63	8/ 2/63	>64
W 502	W-18 Va_2	T	1/31/63	1/31/63	32
	HeLa	T*	1/31/63	8/ 2/63	32
	W-18 Va_2	T	8/ 8/63	8/ 8/63	32
				8/20/63	32
W 510	HeLa	T*	3/ 1/63	3/ 1/63	32
	HeLa + SV_{40}§	T	3/ 1/63	6/21/63	32
W 512	HeLa	T*	3/ 7/63	3/ 7/63	<4
	W-18 Va_2	T	3/ 7/63	8/27/63	<4
	W-18 CM	N	3/ 7/63		
W 516	WI-38 Va_2§	T	3/14/63	3/14/63	8
	HeLa	T*	3/14/63	6/14/63	8
	WI-38	N	3/14/63		
W 521	W-18 Va_2	T	3/29/63	3/29/63	4
	HeLa	T*	3/29/63	4/15/63	4
				8/ 5/63	<4
W 524	W-18 Va_2	T	4/26/63	4/26/63	8
				7/25/63	4
W 530	W-18 Va_2	T	6/ 4/63	6/ 4/63	32
	W-18 Va_2	T	7/ 5/63	6/19/63	16
				7/ 5/63	32
				7/18/63	32
W 536	W-18 Va_2	T	6/27/63	6/27/63	<4
	W-18 Va_2	T	7/25/63	7/25/63	8
				8/15/63	8
W 540	W-18 Va_2	T	7/11/63	7/11/63	<4
				7/17/63	<4
				9/ 6/63	<4
W 531	W-18 Va_2	T	6/ 4/63	6/ 4/63	4
				6/21/63	<4
				9/ 5/63	<4
				9/19/63	<4

† T = Human transformed by SV_{40}; N = normal human diploid; T* = human tumor cell line.

‡ Reciprocal of highest dilution giving 3+ fixation against 4-8 units of hamster SV_{40} tumor antigen.

§ Bearing infectious virus.

transplantation antigens encountered by the human host is very large, and it would be surprising if an antibody against one specific complement-fixing antigen could be detected under these circumstances. Responses following autologous implantation would help to eliminate this problem. The sensitivity of the actual complement-fixation also may have been questioned in connection with negative findings obtained with human sera. However, the presence of CF antigen, demonstrated by means of fluorescent antibody technique(9), in all cells of SV_{40}-transformed human cultures indicates that sensitivity of the present test should not be considered as a factor in the negative results obtained. Finally, the negative findings may have been due to the relatively small numbers of cells in the implant, and therefore small antigenic mass and/or the short time of the transplant's sojourn in the patient's tissues. However, it is interesting to note that such patients already showed a local cellular reaction at the edge of the nodule at 7 days when it was surgically removed and in the few patients whose nodules were not removed the transplant was soon rejected. Furthermore, a few patients who had received 2 transplants several months apart also failed to develop specific antibodies. These negative findings under the conditions of transplantation in human patients are not surprising in view of the importance of tumor mass for CF antibody production and the loss of antibody on surgical removal of the tumor in the hamster(1).

The second interesting finding in the tests of patient's sera was the presence of antibody titers as high as 1/64 against not only the SV_{40} tumor antigen but against other hamster tumors and even normal hamster tissues. The fact that in every such patient the pre-transplant titer was just as high indicated that these antibodies were not the result of the transplant inoculation. The obvious explanation is that certain individuals have antibodies against either some species specific antigen present in hamsters or some common antigenic contaminant of hamsters' tissues. The variations in serum titers against different hamster antigens also suggest that the amount of this antigen in a tumor or organ may vary from one sample to another. Although the nature of this antigen has not been investigated, its presence complicates the interpretations of any findings of CF antibodies in man when crude antigens from a hamster source are being used and reemphasizes the need for pre- and *post-facto* sera and proper control antigens in serological tests using materials from different animal species.

The chief significance of these studies lies in the demonstration that a specific new antigen in SV_{40}-transformed cells is produced just as efficiently in human cells as in those of laboratory animals. The antigen in the transformed human cells reacts with antibody produced in the hamster by an SV_{40} hamster tumor and persists in the cells during their *in vivo* sojourn in man and in spite of a lack of demonstrable virus. This suggests that specific CF antibodies produced in laboratory animals by their own virus-induced tumor antigens might be capable of demonstrating similar CF antigens in any human tumors induced by that same virus.

Summary. Human fibroblast cultures transformed *in vitro* by SV_{40} virus contain a CF antigen which reacts with antibody in sera of hamsters bearing SV_{40}-induced tumors. This specific antigen persisted in the transformed human cells after 2 transplant passages through human volunteers. Cancer patients in whom SV_{40}-transformed human cells had produced nodules failed to develop demonstrable CF antibodies against the SV_{40} tumor antigen. Certain patients, however, were shown to have definite titers of CF antibodies against different types of hamster tumors and against normal hamster tissue antigens; these antibodies were not related to the inoculation of the transformed cells.

1. Black, P. H., Rowe, W. P., Turner, H. C., Huebner, R. J., Proc. Nat. Acad. Sci., 1963, v50, 1148.

2. Habel, K., Eddy, B. E., Proc. Soc. Exp. Biol. and Med., 1963, v113, 1.

3. Jensen, F., Koprowski, H., Pagano, J. S., Ponten, J. A., Ravdin, R. G., J. Nat. Cancer Inst., 1964, v32, 917.

4. Koprowski, H., Ponten, J. A., Jensen, F., Ravdin,

R. G., Moorhead, P. S., Saksela, E., J. Cell. Comp. Physiol., 1962, v59, 281.

5. Jensen, F., Koprowski, H., Ponten, J. A., Proc. Nat. Acad. Sci., 1963, v50, 343.

6. Gerber, P., Science, 1963, v140, 889.

7. Pagano, J. S., Crichlow, R. W., Jensen, F., Cornog, J. L., Ravdin, R. G., Koprowski, H., Proc. Am. Assn. Cancer Res., 1964, v5, 50.

8. Shein, H. M., Enders, J. F., Palmer, L., Grogan, E., Proc. Soc. Exp. Biol. and Med., 1964, v115, 618.

9. Gilden, R., Taguchi, F., Defendi, V., to be published.

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Electrophoretic Analysis of Abnormal Development.* (29742)

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A maternal dietary deficiency of pteroylglutamic (folic) acid is markedly teratogenic in the rat, and furthermore, the types and incidence rate of malformations vary in relation to duration, severity and timing of the deficiency during pregnancy(1). A 48-hour maternal transitory deficiency of this vitamin early in the second week of pregnancy (days 8 and 9), which is a period of rapid differentiation and organogenesis in the rat embryo, will result initially in 100% embryonic malformation and ultimately in embryonic death (2). A reduction in histochemically detectable phosphatase in the neural tube as early as day 9½ was demonstrated(3) as a first sign of incipient malformation. This reduction in phosphatase was followed on day 10 by a partial metaphase block and by reduced numbers and uneven staining of ribosomes as seen with electron microscopy(4). Retardation in the embryonic growth rate was followed by multiple frank malformations on days 11 and 12 and finally by cessation of embryonic heartbeat by the end of the second week(2) of the 3-week gestation period. Intensive study of several organs and organ systems(5) has demonstrated that such rates of development were unequally retarded and that this asynchronism was fundamental to the genesis of structural malformations. The object of the research reported here was to ascertain whether there were similar changes in the multiple molecular forms of enzymes associated with the development of structural malformations in the mammalian embryo.

Methods. A purified diet(2) lacking pteroylglutamic acid (PGA) but containing 20 mg% of the antagonist 9 methyl-PGA was fed on days 8 and 9 of gestation to pregnant, Long-Evans rats which had been inseminated by males of the same strain. The period of deficiency was terminated by feeding a diet which lacked the antagonist but which contained a high level of the crystalline vitamin. On days 10, 11, 12 and 13 of pregnancy embryos were removed from the uteri of PGA-deficient mothers for comparison with normal controls of the same gestational age. Homogenates of these embryos were subjected to zone electrophoresis in starch gel by methods previously described (6). The multiple molecular forms of phosphatases were visualized by standard biochemical means(7), and a semiquantitative representation of staining intensity and band width as observed in the starch zymograms (8) is depicted diagrammatically in Fig. 1 and 2.

Results and discussion. At pH 8.2 a maximum of 4 molecular forms of phosphatase were detected (Fig. 1) with Na alpha naphthyl acid phosphate as substrate in conjunction with the diazo salt Blue RR. At day 10, two forms of the enzyme family (II and IV) were present in both control embryos and in those from mothers maintained by the PGA-deficient diet for the 2 previous days. By day 11 the controls had formed

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