Search for Virus in Human Malignancies. 4. Tests of Human Neoplastic Tissues in Sex-Segregated Mice.* (30796)

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Studies in these laboratories during the past several years have been directed toward detection of an infectious principle of viral or virus-like quality in controlled studies with tissues of human neoplastic origin. *In vitro* tests(1) in cell cultures and *in vivo*(2,3) tests in hamsters and in mice of the ICR/Ha strain failed to detect a virus, virus-like agent or tumor-inducing principle of etiologic significance. There was considerable incidence of spontaneous mammary carcinoma in the mice and this could be reduced drastically by early segregation of the sexes so as to prevent pregnancy(2,3). The present report summarizes the findings in continued studies of human neoplastic specimens employing as test animals mice which had been sex-segregated so as to take advantage of the reduction in spontaneous mammary tumor. Part of the human specimens were tested either as crude extracts or after treatment with fluorocarbon to remove hypothetical inhibitor such as antibody. Additionally, a portion of the specimens were treated according to a procedure described by Burton, Friedman and associates(4) which has been reported to be used successfully for processing specimens of neoplastic tissues of men and mice so as to cause appearance of primary parotid, mammary, adrenal and soft tissue tumors and leukemia upon inoculation into C3H(f) mice.

Materials and methods. Clinical specimens. Tissues from cases of cancer in man were obtained from the Department of Hematology, Children's Hospital of Philadelphia; from Montgomery Hospital, Norristown, Pa.; and from Albert Einstein Medical Center, Philadelphia. The methods used for collecting human specimens were described earlier (1) and the kinds of tissue which were included are shown in Table I in the text. The tissues were stored frozen at −70°C or at −20°C prior to being processed for testing. In certain instances, "normal" tissue from the same cancer patient or from non-cancer patients was included for control purposes. *Processing of specimens.* Tissues were homogenized for 10 minutes at 16,000 rpm in an Omnimix cup held in an icebath employing sufficient sterile Hanks' balanced salt solution (HBSS) to give a 15% suspension. The crude supernate obtained following centrifugation for 10 minutes at 1500 rpm was inoculated into mice without further treatment or following 2 successive extractions with Freon 113 (fluorocarbon) as described previously(1). The Burton-Friedman type extracts were prepared precisely as described by Burton *et al*(4). To insure accurate duplication of the method, one of us (A.J.G.) visited the laboratories of Drs. Burton and Friedman on several occasions to observe procedures and to perform extractions. *Inoculation and observation.* The ICR/Ha strain mouse was free of polyoma virus infection and was random bred genetically in these laboratories. Newborn mice less than 20 hours old were inoculated with 0.1 to 0.2 ml of specimen in the subcutaneous space of the dorsal nuchal area. A portion of animals was held as uninoculated controls. All animals were sex-segregated at time of weaning on the twenty-first day of age and were retained as virgins throughout the experiments. The animals were kept in isolation in filter cages during the first 60 to 90 days and all manipulations involved in care and observation were as described previously(2). It is worthy of emphasis that the animals were palpated for tumors at least once each week and, in addition, each cage was examined twice daily for dead animals. All tumors or other tissues which appeared abnormal

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were harvested at time of spontaneous death of the animal or on termination of the experiment and were processed for histomorphologic examination. The experiments were terminated 480 to 518 days after the animals were inoculated and the control animals were always sacrificed at the same time as the corresponding test group.

Results. Table I summarizes, for inspection, details of the occurrence of neoplasia in mice of the various groups. There was nothing remarkable except that the tumors appeared very late, at 250 to 500 days of age when they commonly occur spontaneously in mice. Many different kinds of neoplasia were found, predominantly mammary carcinoma, and the occurrence of neoplasia appeared to be at random with respect to inoculum.

TABLE I

TABLE I. Occurrence of Neoplasia in Experimental and Control Mice According to Sex (M/F) in Experiments 1 and 2.

Clinical material			OBSERVATIONS IN MICE (MALE/FEMALE)																								
							Numbers of animals with histologic type of neoplasia (M/F)																				
Type of neoplasia	Specimen		No. Weaned	No. Non-Specific Deaths	No. with Cancer	Age when positive x 100 (± 25 days)	Mammary carcinoma	Pulmonary carcinoma	Malignant *** lymphoma	Leukemia	Fibrosarcoma	Histiocytoma	Reticulum cell sarcoma	Hemangioma	Plasma cell tumor	Intestinal carcinoma	Epidermoid carcinoma	Anaplastic carcinoma	Hepatoma	Myxosarcoma	Mesothelioma	Osteogenic sarcoma	Lipoma	Undifferentiated tumor
	Organ	Treatment																									
(Dittos = same case)			M/F	M/F	M/F	M/F																					
EXPERIMENT 1. (Crude & Fluorocarbon)																											
Leukemia, type unknown	Spleen	Crude	15/17	9/1	1/5	5/3-5	/3						1/2														
" " "	"	Fluoro.	23/20	5/3	/1	/5													/1								
" " "	Liver	Crude	13/17	6/1	/3	/3-4			/3																		
" " "	"	Fluoro.	27/18	4/3	1/2	5/3.5	/1		1/				/1														
Leukemia, acute lymphoc.	Marrow	Crude	4/2	1/1	1/	5/							1/														
Leukemia, acute lymphoc.	Spleen	Crude	20/14	14/3	/1	/5			/1																		
" " "	Liver	"	6/																								
Leukemia, acute lymphoc.	Brain	Crude	24/16	11/	1/3	5/2.5-5	/1						/1							1/			/1				
" " "	"	Fluoro.	20/18	14/5	1/4	4.5/4-5	/4				1/																
Leukemia, acute lymphoc.	Marrow	Crude	22/25	13/2	2/2	5/2.5-5	/1		/1								1/				1/						
" " "	"	Fluoro.	20/26	15/7	/1	/3																					
Leukemia, acute lymphobl.	Brain	Crude	14/10	14/2	/1	/3.5	/1																				
" " "	"	Fluoro.	26/9	25/3	/1	/5			/1																		
" " "	Marrow	Crude	16/10	15/3	/2	/5			/2																		
" " "	"	Fluoro.	26/29	14/8	3/6**	5/5	/3		3/4		/1																
" " "	Nodes	Crude	28/13	14/3	1/1	4.5/5	/1				1/																
" " "	"	Fluoro.	16/13	10/5	/1	/5					/1																
" " "	Node	Crude	24/13	11/1	2/1	4.5-5/5	/1		2/																		
" " "	"	Fluoro.	21/17	10/3	/1	/5															/1						
Leukemia, acute granulo.	Brain	Crude	24/23	13/1	2/3	3.5-4/4.5-5	/1		/2				2/														
" " "	"	Fluoro.	14/12	4/1	1/2*	3.5-5/5	/1		1/2																		
Carcinoma, lung	Lung	Crude	17/16	14/4	1/2	3.5/5	/1	1/	/1																		
Carcinoma, lung	Lung	Crude	15/16	7/1	1/2	5/4.5	/1	/1					1/														
Fibrosarcoma	Muscle	Crude	11/8	2/	/1	/5	/1																				
Carcinoma, lung	Norm. Lung	Crude	24/18	19/2																							
Carcinoma, lung	Norm. Lung	Crude	20/26	4/5	1/1	4/5			/1		1/																
Normal	Spleen	Crude	47/46	33/9	1/5*	5/3.5-5	/3		/1	/1			1/1														
"	"	Fluoro.	5/4	1/1	/2	/5			/1				/1														
Control - 11/15/63	Hanks' BSS	Fluoro.	59/65	30/11	*2/9	5/3.5-5	/3	1/1	2/2		/1			/1					/1								
Control - 11/22	" "	Fluoro.	55/54	43/11	2/11*	4.5-5/3.5-5	/7		1/5					/1			1/										
Control - 2/8	" "	Nil	33/23	27/12	/2	/5		/1						/1													
Control - 2/6	Nil	---	35/24	28/11	/6	/4-5	/3	/2		/1																	
Control - 2/13	Nil	---	21/22	13/8	1/	5/	1/																				
Control - 5/10	Nil	---	21/29	11/8																							
Control - 5/29	Nil	---	21/19	14/7	1/2	5/4.5-5	/1		/1				1/														
Control - 6/12	Nil	---	21/19	16/5	/3	/4.5-5	/2																				/1
Control - 9/18	Nil	---	36/52	19/11	/3	/3.5-5	/2		/1																		
Control - 9/20	Nil	---	80/91	43/18	/14	/3.5-5	/11						/3														
Control - 11/27	Nil	---	47/27	28/4																							
EXPERIMENT 2. (Burton-Friedman)																											
Leukemia, acute lymphoc.	Spleen	B-F	11/7	2/1	4/1	4.5-5/5	1/	1/1				1/	1/														
" " "	Liver	B-F	12/3	3/1	/1	/5						/1															
Carcinoma, stomach	Liver	B-F	20/20	3/2																							
Carcinoma, small intest.	Intest.	B-F	15/8	8/2	1/	5/					1/																
Carcinoma, pancreas	Liver	B-F	20/17	9/3	/1	/2.5												1/									
Carcinoma, rectum	Rectum	B-F	20/10	5/4																							
Carcinoma, breast	Breast	B-F	21/16	11/2	1/	5/		1/																			
Carcinoma, lung	Lung	B-F	15/21	3/3	2/	4-4.5/		1/		1/																	
Carcinoma, lung	Lung	B-F	14/12	4/5																							
Carcinoma, colon	Colon	B-F	10/6	7/	/3	/3-5			/1	/1						1/											
Carcinoma, prostate	Prostate	B-F	17/14	2/	/3	/2.5-5	/1		/2																		
Carcinoma, thyroid	Thyroid	B-F	18/8	2/																							
Carcinoma, generalized	Mixed	B-F	18/5	4/1																							
Neuroblastoma	Brain	B-F	10/6	5/0																							
Normal	Spleen	Crude	12/13	12/2	/3	/3.5-5	/1		/2																		
Normal	Spleen	B-F	11/12	2/2	1/3	4.5/3.5-4.5	/3				1/																
BF gel	Tris buff.	B-F	19/12	6/3																							
Control - 1/18/63	Tris buff.	pH 6.5	26/15	6/1																							
Control - 1/18	Tris buff.	pH 7.5	12/9	3/																							
Control - 1/25	Tris buff.	pH 7.5	6/8																								
Control - 2/1	Tris buff.	pH 7.5	17/15	4/5	/2	/3.5-5	/1												/1								
Control - 1/9/63	Nil	---	28/12	6/3	/1	/5	/1																				
Control - 1/16	Nil	---	29/24	11/4	/1	/5	/1																				
Control - 1/23	Nil	---	24/31	6/6	/6	/5		/2	/2						/1		/1										
Control - 1/30	Nil	---	31/25	11/7																							
Control - 2/1	Nil	---	3/8	1/4	/1	/3			/1																		

*Two different kinds of neoplasms in one mouse.
**Two " " " " " two mice.
***Exclusive of reticulum cell sarcoma.

TABLE II. Summary, Occurrence of Neoplasia, All Types, in Test Mice Compared with Controls in Experiments 1 and 2.

Group	No. weaned ♂	No. weaned ♀	No. survived* ♂	No. survived* ♀	Neoplasms in survivors: Male No.	Male %	Female No.	Female %	Both sexes No.	Both sexes %
Exp 1 (crude & fluorocarbon)										
Experimental	446	362	201	301	18	9.0	46	15.3	64	12.7
Control (total)	525	519	196	396	8	4.1	58	14.6	66	11.1
Placebo	243	236	86	185	6	7.0	30	16.2	36	13.3
Uninoculated	282	283	110	211	2	1.8	28	13.3	30	9.3
Exp 2 (Burton-Friedman)										
Experimental	221	153	153	129	8	5.2	9	7.0	17	6.0
Control (total)	218	184	150	147	1	0.7	17	11.6	18	6.0
Placebo	103	84	70	71	1	1.4	8	11.3	9	6.4
Uninoculated	115	100	80	76	0	0	9	11.8	9	5.8
Total, all groups	1410	1218	700	973	35	5.0	130	13.4	165	9.9

* Includes animals which died with or were sacrificed with neoplasia.

Table II shows that among 2628 weaned animals initially included in the group, 955 (36%) died without detected malignancy while 1673 (64%) survived and either remained normal or developed tumor during the period of observation. Among the total group of animals which received crude or fluorocarbon-treated material, the neoplasia rate was 12.7% compared with 11.1% for the controls. The rate was 6.0% in the experimental and control groups in tests of specimens processed by the Burton-Friedman method. Additionally, the tumor rate was roughly the same in animals which received placebo compared with uninoculated controls.

There was far greater incidence of neoplasia in female compared with male mice, *viz.*, 13.4% and 5.0%, respectively. As shown in Table III, this was due, primarily, to a much higher rate for mammary carcinoma and malignant lymphoma among females compared with males and was evident both in Experiments 1 (crude and fluorocarbon extracts) and 2 (Burton-Friedman extracts).

TABLE III. Summary, Occurrence of Neoplasia, According to Kind of Neoplasm and Sex, in Experimental and Control Mice in Experiments 1 and 2.

Type of neoplasia	Exp 1 ♂	Exp 1 ♀	Placebo 1 ♂	Placebo 1 ♀	Uninoc'd 1 ♂	Uninoc'd 1 ♀	Total 1 ♂	Total 1 ♀	Exp 2 ♂	Exp 2 ♀	Placebo 2 ♂	Placebo 2 ♀	Uninoc'd 2 ♂	Uninoc'd 2 ♀	Total 2 ♂	Total 2 ♀
	Number of neoplasms according to inoculum and sex															
	Experiment 1 (crude and fluorocarbon)								Experiment 2 (Burton-Friedman)							
Mammary carcinoma	0	21	0	13	1	19	1	53	1	1	0	5	0	2	1	8
Pulmonary carcinoma	1	1	1	2	0	2	2	5	3	1	0	0	0	2	3	3
Malignant lymphoma	7	17	3	10	0	2	10	29	0	3	0	2	0	3	0	8
Leukemia	0	0	0	1	0	1	0	2	1	1	0	0	0	0	1	1
Fibrosarcoma	2	2	1	1	0	0	3	3	1	0	1	0	0	0	2	0
Histiocytoma	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1
Reticulum cell sarcoma	5	4	1	2	1	3	7	9	1	0	0	0	0	0	1	0
Hemangioma	0	0	0	2	0	0	0	2	0	0	0	0	0	1	0	1
Plasma cell tumor	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Intestinal carcinoma	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Epidermoid carcinoma	1	0	1	0	0	0	2	0	0	1	0	0	0	0	0	1
Anaplastic carcinoma	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
Hepatoma	1	1	0	1	0	0	1	2	0	0	0	0	0	0	0	0
Myxosarcoma	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Mesothelioma	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Osteogenic sarcoma	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Lipoma	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Undifferentiated tumor	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
Totals	18	49	7	32	2	28	27	109	8	9	1	8	0	9	9	26

There was considerable difference in the overall tumor rate in the 2 experiments. The rate in the inoculated and control mice in Experiment 1 was roughly twice that for the 2 groups in Experiment 2. Overall rates for tumor occurrence in the placebo and uninoculated control groups in each experiment were not significantly different. The difference in amount of neoplasia between male experimental and control animals in Experiment 2, *viz.*, 5.2% *vs* 0.7%, was regarded as reflecting random variation in occurrence of tumors of diverse types among the animals.

Discussion. Consistent with earlier work (1-3) the findings in the present investigation failed to disclose the presence of any virus or virus-like agent or tumor-inducing principle in the malignant neoplastic tissues of human origin. Sex-segregation did not bring about any apparent improvement in ability to detect the hypothetical virus or viruses using ICR/Ha mice. Additionally, the experiments failed to confirm the earlier findings of Burton, Friedman *et al*(4) of induction of primary parotid, mammary, adrenal and soft tissue tumors and leukemia following inoculation of human tumor tissues which had been processed by the Burton-Friedman method.

Except for leukemia, the diverse tumor types reported by Burton, Friedman *et al*(4) to occur in mice inoculated with human tumor processed by the Burton-Friedman method were suggestive of the tumors caused by polyoma virus which is a common contaminant of mouse stocks. Early query revealed that the mice in the St. Vincent's Hospital colony which were used by Burton, Friedman and their co-workers had not been tested for polyoma virus infection. Accordingly, sera from 23 mice from the St. Vincent's colony were tested by us for presence of hemagglutination-inhibitory antibody against polyoma virus following treatment with receptor destroying enzyme to remove non-specific inhibitors. The sera were found to have polyoma antibody titers as follows: 11 positive at 1:200; 1 positive at 1:100; 1 positive at 1:50; and 11 negative at 1:50. Similar tests of sera from more than 1000 mice of the ICR/Ha colony maintained here revealed no occurrence of polyoma antibody. The failure to confirm the findings in the Burton *et al* report(4) and the demonstration of indigenous polyoma infection in the St. Vincent's colony strongly suggested that the tumors in mice reported by Burton *et al* were not caused by factors of human tumor origin, even though the mouse strain used in our studies (ICR) differed from theirs [C3H(f)]. Tests for polyoma tumor complement-fixing antigen in their mouse tumors might provide a more definitive basis for establishing the etiology of tumors in the mice.

There was considerable difference in overall tumor incidence in the 2 experiments, *viz.*, 12.7% and 11% for the experimental and control groups, respectively, in Experiment 1 compared with 6% and 6% for Experiment 2. Such variation is not uncommon in investigations of this sort. There was a far greater incidence of neoplasia in female mice, 13.4% than in male mice, 5.0%. This was due to a much higher rate of mammary carcinoma and malignant lymphoma among females compared with males. The higher rate for mammary tumors in females was expected. The higher rate of malignant lymphoma among females may be related to the greater length of survival and risk among females compared with males. The shorter survival time for males compared with females was due primarily to death from fighting. Sex-segregation in the present study did not appear to reduce the incidence of mammary carcinoma to the extent achieved earlier (3). The overall rate for mammary carcinoma for all groups of both sexes in the present series was 3.7% (63/1673) compared with 6.6% (71/1083) in non-sex-segregated mice(2). This is in contrast to the approximate 8-fold reduction in mammary tumor resulting from sex-segregation as recorded in the third report(3) of this series, *viz.*, 8% (155/1931) *vs* 1% (2/213).

A number of workers have recorded(4-28) presence of viruses, interfering agents, or virus-like structures in primary tumor or in cell cultures of human neoplastic tissues. None of these have been established to date as being of etiologic significance in human cancer. Others(29-32), like ourselves,

have failed to demonstrate any agent of significance in human tumor. Virus-dependent tumors should have virus present and recovery would depend upon supplying the satisfactory cellular substrate for its demonstration. Virus-initiated but independent tumor may no longer contain sufficient viral genome to replicate infectious virus and recovery might depend upon chance occurrence of superinfection or carrier virus in the tumors. None of the necessary criteria for satisfactory virus demonstration and proof of etiologic relationship have been met to date. Negative findings such as recorded in the present and previous(1-3) reports emphasize the difficulty in recovering virus in human malignancy but do not exclude the possibility. Furthermore, positive results in studies such as these would necessarily depend upon susceptiblity of the mice to the human tumor agent or agents if they existed.

Summary. A total of 38 neoplastic tissue preparations from a variety of human cancers were tested for presence of a tumor-inducing principle in newborn ICR/Ha mice. The mice were sex-segregated to prevent pregnancy and to limit occurrence of spontaneous mammary carcinoma. The malignant tissue specimens were tested as crude extracts, after fluorocarbon extraction, or following treatment by the Burton-Friedman method which was reported to make possible tumor induction in mice. There was no evidence for a tumor-inducing principle in any of the specimens tested. Numerous spontaneous neoplasias were observed and mammary carcinoma and malignant lymphoma were most commonly present.

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