ever, to establish the feasibility of the PCA test in study of autoantibodies.

It was seen by Snapper and Grunbaum that diiodotyrosine and thyroxine produced no inhibition in rabbit sera containing thyroid heteroantibodies(22). The fact that diiodotyrosine, triiodothyronine, and thyroxine do not neutralize the human thyroid autoantibody suggests that antibody is not simply directed against antigenic determinants with configurations similar to these substances. The further fact that human thyroglobulin completely neutralizes the antibody suggests that the antibody detected by these PCA experiments is directed against some other portion of the globulin structure.

Summary. 1. Passive cutaneous anaphylaxis of the guinea pig has been successfully used to study thyroid autoantibodies of rabbit, guinea pig, and man. 2. The thyroid autoantibody proved to be specific for the homologous antigen (thyroid extract and purified derivative) and did not give cross reactions with thyroid extracts of other species. 3. Five human sera with high titer of thyroid autoantibody were studied. The antibody was completely neutralized by human thyroid extract or human thyroglobulin, but was not significantly affected by any of a variety of other organ extracts or of iodinated amino acids, again indicating the specificity of this type of antibody.

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Agent in Ak Leukemic Tissues, Not Sedimented at 105,000 g, Causing Neoplastic and Non-neoplastic Lesions.*† (24364)

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Following earlier work suggesting that the leukemia agent of Gross(1) may be analogous to the transforming principle of microorganisms(2), an experiment was set up to identify

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the fraction of the cell which possesses viral activity. Ak leukemic tissues were fractionated into a) mitochondria and microsomes, b) nuclei, and c) supernatant fluid, and these injected into newborn mice. Unfortunately, all but the supernatant fluid were contaminated. However, the supernatant fluid resulting from 105,000 g centrifugation, caused development of an amazing number and type of tumors and non-neoplastic changes in diverse organs, not seen in buffer injected and uninjected controls and in tens of thousands of Ak mice studied by one of us earlier, including the series in which newborn mice were injected with Ak leukemic extracts(2).

Materials and methods. Mice. AkR and Rf mice were obtained from Jackson Memorial Laboratory, Bar Harbor, Me.; C3H (foster-nursed subline) from Dr. Ludwik Gross. All were bred in our laboratory. Purina chow and water were given *ad lib.*, and vegetables once a week. Breeding females were given hemp seed once a week, and females with litters bread and milk daily. Weanling mice also received bread and milk for another 7 to 14 days. Terramycin was given in drinking water when necessary to combat infection. Newborn mice (less than 22 hours old) were injected subcutaneously with 0.03-0.05 ml of the various materials. For identification, the tails of mice receiving infective materials were ligated immediately; tails of control mice were left intact. Ear-marking and toe-clipping were done at weaning.

Material injected. AkR leukemic tissues were obtained from two 7-month-old AkR female mice, each with spontaneous thymic and generalized lymphoma, and carried separately through 3 serial subpassages. Each subpassage yielded 100% takes. For fractionation, lymph nodes (excluding cervical nodes), spleens, thymuses and thigh tumors (produced by intramuscular grafts) were collected and pooled from the third subpassages. (Liver and brain were not included). Fresh leukemic tissues were passed through a 30-mesh steel screen and suspended in isotonic phosphate buffer (with calcium and antibiotic‡ added) at pH 7.4, making a 20% dilution of tissue by weight. The suspended material was homogenized by the nitrogen pressure method of Hunter and Commerford (to be published) (900 lb pressure/sq. in./20 min.) 4 times. An aliquot of this homogenate was removed for injection, and the remainder subjected to nitrogen pressure for a fifth time. A few drops of the homogenate were examined microscopically for the presence of intact cells, none were seen. Phosphate buffer was added to the homogenate to make a final 10% dilution of tissue by weight, and the material spun in the International Centrifuge at 2000 rpm/10 min. The supernatant fluid was removed and the sediment, the nuclear fraction, resuspended in phosphate buffer (20% dilution). The supernatant fluid was placed in the Spinco Centrifuge (Model L) in 10 ml aliquots, spun at 105,000 g for 35 minutes, and the final supernatant fluids collected and pooled. The sediment, the mitochondrial-microsomal fraction, was resuspended in phosphate buffer (20% dilution). Materials were handled aseptically and kept chilled throughout all procedures. Fractionation was done in Jan. 1957. All fractions, and buffer, were sealed in ampoules in quantities of about 0.8 ml and stored at −60 to −70°C. Injections were made during subsequent 12 months' period, as newborn mice became available. Fractions were injected immediately after thawing.

Results and comments. Pre-weaning mortality. Pre-weaning mortality was higher in AkR mice which received the 105,000 g supernatant fluid (63%) than in uninfected AkR controls (47% in buffer injected and 32% in uninjected mice). Whether this is related to viral infection has not been ascertained. In Rf mice injected with 105,000 g supernatant fluid, pre-weaning mortality was 28% as compared with 33% of buffer injected controls. In groups receiving the homogenate, mitochondrial-microsomal and nuclear fractions, the mortality was very high, notably in AkR mice (71-73%) and the fate of survivors is included here merely for the sake of completeness.

Tumors. Of 32 AkR mice injected with the 105,000 g supernatant fluid from AkR leukemic tissues, 87.5% developed tumors of

‡ 400,000 U Penicillin G procaine and 30 U Streptomycin sulfate/ml of buffer.

TABLE I. Tumor (T) and Leukemia (L) Incidence in AkR and Rf Mice Injected with 105,000 g Supernatant Fluid and Other Fractions from AkR Leukemic Tissues.

Group	Induced with	No. in group	Disease incidence (days of age)* 90-180 T	90-180 L	90-180 Neg.	181-240 T	181-240 L	181-240 Neg.	241-360 T	241-360 L	241-360 Neg.	360- T	360- L	360- Neg.	Total T No.	Total T %	Total L No.	Total L %	Total Neg. No.	Total Neg. %
	AkR recipients																			
1	105,000 g supernatant	32	22	0	1	3	1	1	3	4	1	0	0	0	28	87.5	5	15.8	2	6.3
2	Other fractions	8	1	1	0	0	0	0	0	5	0	1	2	0	2	25.0	8	100.0	0	
3	Controls	59	1	2	11	2	5	18	0	5	20	0	0	0	3	5.1	12	20.3	46	78.0
	Rf recipients																			
4	105,000 g supernatant	39	4	1	8	1	1	5	0	0	2	1	4	14	6	15.4	6	15.4	29	74.4
5	Other fractions	41	1	1	11	0	1	5	1	3	1	0	0	18	2	4.9	5	12.2	35	85.4
6	Control	32	0	0	5	0	0	2	0	1	3	0	2	19	0		3	9.4	29	90.6

* Only newly discovered lesions found at autopsy are indicated at each time interval.

Group 1. One negative is still alive. Three mice living longer than 210 days had both tumors and leukemia. Group 2. Pool of 2 groups receiving homogenate and mitochondrial-microsomal fraction. Two mice had both tumors and leukemia. Group 3. Some uninj., some buffer inj. The 3 mice with tumors were uninj. The 2 in the 181-240 day group had both tumors and leukemia. Forty-one mice in this group are still alive, between 5 and 10 mo of age. Group 4. Two mice in the first age groups had both tumors and leukemia. Group 5. Pool of 3 groups receiving homogenate, mitochondrial-microsomal and nuclear fractions. One animal had both tumors and leukemia.

various sorts at frequencies indicated in Table I, as compared to 5.1% in the controls. Most tumors occurred within 6 months. Many were discovered at 88-128 days of age. Of special interest is occurrence of similar tumors in 3 control mice. All 3 were uninjected and caged in the same area of the room as the mice infected with the supernatant fluid, but were never in physical contact with them. This is an amazing finding, in view of the absence of similar tumors in tens of thousands of AkR mice studied by one of us in the past few decades. Another 2 saline injected AkR mice (controls for another series) likewise developed salivary gland tumors. These observations suggest that this tumor virus spreads by natural means. A similar conclusion has recently been reached by Sarah Stewart (personal communication).

Tumors also developed in 6 of 39 (15.4%) Rf mice injected with the 105,000 g supernatant fluid, and in 2 of 41 (4.9%) Rf mice injected with other fractions. None was noted in 32 controls, and in much larger numbers of Rf mice used in different experiments. In fact, their occurrence has never been recorded in Rf mice.

Leukemia. Leukemia occurred in lesser frequency among the AkR mice injected with the 105,000 g supernatant fluid than among the controls. This is undoubtedly due to death of many mice by tumors before the "leukemia age" (most occurred within 3-6 months). Leukemia may have been induced in mice receiving fractions containing particles larger than those present in the 105,000 g supernatant fluid, but the number of mice injected and incidence of leukemia under 6 months of age were too small for definite conclusions.

Character of tumors induced. Twenty-five AkR mice which received 105,000 g supernatant fluid have been thoroughly studied histologically. Table II lists tumorous and pre-tumorous changes. From this, a triad of lesions emerges which are almost invariably associated: salivary gland (neck) tumors, reticulum cell sarcomas of the thymus (Fig. 1), and adenomatoid pre-tumorous changes in tubules of the kidney cortex (Fig. 3). Common, but less frequent, are non-neoplastic

TABLE II. Types of Tumors and Non-neoplastic Changes Seen in AkR Mice Receiving 105,000 g Supernatant Fluid from AkR Leukemic Tissues.

Sex	Age at death, days	Parotid gland,	Thymus			Kidney		Lung		Thyroid		Tracheal gland		Mammary gland			Cavernous congestion	Bone,
		t	r	rl	l	p	sa	ca	m	p	t	p	t	p	sa	ca		sa
♂	106	×				×							×					
♀	113	×	×			×					×					×		
♂	116	×				×												
♂	118	×				×						×						
♂	119	×	×			×				×					×		×	
♂	119	×	×			×		×								×		
♂	126	×	×			×		×				×						
♀	128	×	×			×	×					×				×	×	×
♂	128	×	×			×									×	×		
♀	133	×	×			×			×			×				×	×	
♂	133	×		×		×				×			×			×	×	
♂	135	×	×			×	×				×		×					
♀	148	×	×			×									×		×	
♂	150	×	×			×										×		
♂	150	×	×			×			×				×				×	
♂	154	×	×			×		×		×								×
♀	157	×	×			×										×	×	
♂	158	×	×			×												
♀	159	×	×			×			×					×			×	
♂	172	×				×		×				×						
♀	226	×		×										×				
♀	226	×		×		×								×			×	
♀	275	×			×													
♀	299	×	×															
♂	344	×			×	×												

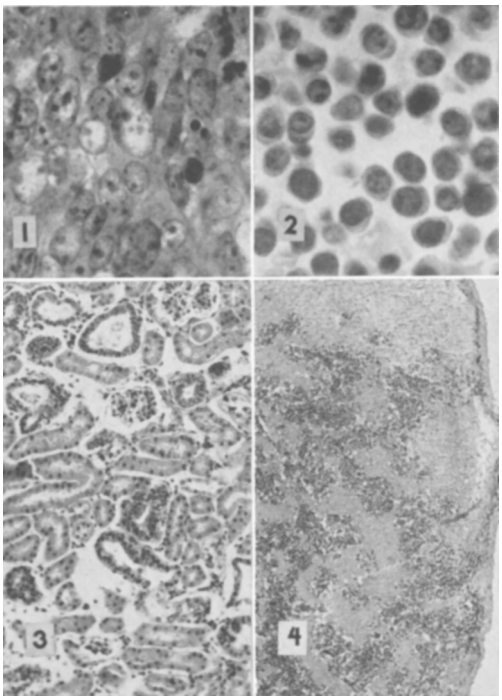
Abbreviations: t = tumor; r = reticulum cell tumor; rl = reticulum cell tumor and lymphoma; l = lymphoma; p = pretumorous proliferative changes; sa = sarcoma; ca = carcinoma; m = metastatic carcinoma.

changes including a marked cavernous congestion of vessels, predominant in the spleen (Fig. 4), of the type described in the presence of hypervolemia(3). The spectrum of tumors noted was, in general, similar to that found by Stewart *et al.*(4) in mice that had been injected with extracts of tissue cultures of salivary gland tumors induced by virus. Adrenal tumors, hemangiomas and hepatomas were not seen in this series. Most animals autopsied with tumors were underdeveloped and emaciated, weighing approximately 14-20 g. Their spleens and lymphnodes were atrophic.

The same triad of changes was also seen in 3 Rf and one AkR control mouse. We were not aware of these microscopic changes early in the course of these experiments, sections were not taken systematically, and lesions of microscopic dimensions remained undetected.

Effect of freezing. Due to spatial limitations, it took about 12 months to complete the injections of fractions in newborn mice, during which time the fractions were kept frozen in sealed ampoules at −60 to −70°C. There was no evidence of deterioration of the agent in the 105,000 g supernatant fluid during this period. The same triad and other changes occurred at about the same frequency throughout this period of study.

Pathology. The articles of Gross(1) and of others cited by him illustrate abundantly the character of the salivary gland and most other tumors. Fig. 1-4 show lesions that are less well understood. Fig. 1 and 2 contrast the reticulum cell sarcoma of the thymus with the common lymphosarcoma of the thymus. There is this sharp biologic difference between them: The reticulum cell tumor causes a marked enlargement of the thymus which remains circumscribed and encapsulated. Lymphosarcoma, on the contrary, is ill-defined and usually invades the pericardium and pleura causing sero-sanguineous pleuro-pericardial effusion, and extends into the surrounding tissues of the mediastinum, most commonly along the vertebral column. However, reticulum cell sarcoma can metastasize



All sections were stained with hematoxylin and eosin.

FIG. 1. Reticulum cell sarcoma of thymus. × 650.
FIG. 2. Lymphosarcoma of thymus. × 900.
FIG. 3. Hyperplastic and pre-neoplastic changes in renal tubules. × 100.
FIG. 4. Cavernous congestion in spleen. × 55.

with formation of discrete tumor nodules.

Fig. 3 illustrates the hyperplastic and pre-neoplastic changes in the tubules of the renal cortex. Almost every section of both kidneys is riddled with such changes. Characteristically, they consist of a great increase in the number of cells lining the tubules, with piling up of cells and formation of infolding in the dilated tubules, terminating in development of papillary cystadenomas. Many of these cells possess giant nuclei. Disseminated lymphoid foci are often present, but they seem to be more of the hyperplastic than the leukemic type. Their relation to virus requires further study. Sarcomas were encountered in the medulla of the kidney in 2 mice.

Fig. 4 illustrates the cavernous congestion of capillaries frequently seen in the spleens and in a few other organs of tumor bearing mice. Such lesions are sometimes confused with vascular tumors. They resemble those encountered with hypervolemia(3).

Nucleic acid content of 105,000 g supernatant fluid. Assay for DNA content of the supernatant fluid by Keck's modification of the Ceriotti indole reaction method(5) revealed 30 μg/ml. This is a surprising finding in view of the known absence of DNA in the cytoplasm of cells(6). Assay for RNA content of the supernatant fluid by Ceriotti's method(7) revealed 295 μg/ml, which is within the range expected for normal cells. It is possible that this DNA represents contamination of the cytoplasm with nuclear DNA due to presumed disruption of some nuclei. It is also possible that the DNA is derived from virus residing in the cytoplasm, or that the level reported here may represent depolymerization products or DNA precursors rather than DNA *per se,* since Keck's method measures *deoxyribose.*

Tumor viruses are NA complexes, and evidence is mounting that NA concentrates of leukemic cells have viral activity. Hays *et al.*(8) described induction of leukemia with NA concentrates prepared in a manner which, in their opinion, could not be withstood by known viruses. Latarjet *et al.*(9) have produced multiple tumors with nucleic acid concentrates derived from leukemic tissues.

Relation of leukemia virus to tumor virus. The development of salivary gland and a few other types of tumors in mice that have been injected with Ak and C3H leukemic extracts has been reported by Gross(1) and confirmed by others(1). Stewart *et al.*(4) injected mice and other species with supernatant fluid of tissue cultures of salivary gland tumors, and noted development of a large number and many types of tumors. The causative agent was designated by them as "polyoma virus." Stewart is inclined to believe that this is the same virus which causes leukemia, originally discovered by Gross, and that in the course of passages and cultivation *in vitro* has either increased in concentration or changed in virulence. The following observations made by us support the opinion that we are dealing with 2 viruses, one causing leukemia, the other the many lesions described here and by Stewart *et al.*(4). We shall designate the leukemia agent as L virus, and the tumor inducing agent as P virus. In our earlier work

(2), Ak leukemic extract injected into a large number of mice produced leukemias, but no tumors. Salivary gland tumors are large, they were looked for, and it is unlikely that they were missed by us. Presently, we have 2 viruses in the laboratory, and the difference between them is striking. In the experiments reported here, the 105,000 g supernatant fluid from Ak leukemic tissues produced tumors and pre-tumorous changes in 87.5% of the injected AkR animals, and no leukemias, suggestive of high concentration of the P virus. In parallel experiments, Dr. Jeana Levinthal has been injecting newborn C3H mice with C3H leukemic extract (originally also derived from Ak leukemia but passed by Gross through C3H mice) producing about 60% leukemias but no tumors. Presently, the relation between L and P viruses remains conjectural. It is possible that P virus is a derivative of the L virus and is of smaller molecular weight, or that it is a contaminant of the Ak strain and is distinct from the L virus.

Origin of P virus. Manifestations of P virus were absent in our Ak colony until after we began working with the C3H Bittner line of mice. The latter is known to develop salivary gland tumors spontaneously (Law, 10). This suggests that the P virus was introduced by use of the C3H Bittner line, and, gaining virulence in the course of animal passages, has become infective for other strains of mice, and, now, also for other species (Sarah Stewart, personal communication). In support of this opinion it should be emphasized that such tumors appeared in 3 Ak controls in the present series, and in 2 controls in another smaller series not reported here indicating spread by natural means. Similar tumors have never been noted before in numerous studies carried out with Ak mice. The kidneys were often sectioned by us, and lesions such as those described would not have been missed. Salivary gland tumors in Ak mice that had been injected with Gross virus were first noted by Dulaney *et al.*(11).

One remarkable feature of the P virus is that, coupled with its high contagiousness, goes its property to become masked. Attempts were made to transplant several strains of salivary gland tumors (some obtained from L. Gross) in well inbred mice with very poor success. If these neoplasms were autonomous, 100% transplantability would be expected in the homozygous strains of origin (C3H or Ak). These findings lead us to suppose that the P virus is of the parasitic type(12) causing a conditioned, and not an autonomous, neoplasm, becoming rapidly masked in its hosts. The present experiments indicate that enrichment or modification by cultivation *in vitro* is not necessary to unmask the P virus. How it spreads under natural conditions still remains to be elucidated.

If the supposition is correct that the Ak strain has been contaminated with the pluripotent P virus, then this virus should not be found in laboratories which are carrying the original Ak strain and not C3H mice, notably the Bittner line. Verification of this supposition is urgently needed; for infection, the 105,000 g supernatant fluid from Ak leukemic tissues may be used.

These developments, the outgrowth of contributions by Gross(1), have opened up many new avenues in tumor virus research. As a working hypothesis, we propose that the P virus is parasitic, highly contagious, has its own genome, and consequently is antigenic in the host which it infects, while the L virus is derived from the cell's genome (is "cytogenic,"12), is not antigenic, does not spread by natural means, and its "infectivity" is analogous to that of the pneumococcus transforming principle, possibly a mere laboratory phenomenon.

Summary. 1) Supernatant fluid from 105,000 g centrifugation of Ak leukemic tissues caused a multiplicity of tumors and a variety of non-neoplastic lesions in 87.5% of AkR mice injected shortly after birth. 2) Evidence reported here, and obtained by others (notably Gross, Stewart *et al.*) suggests that lesions described here, are caused by a pluripotent agent (to be called P virus) which is distinct from that which causes leukemia (to be called L virus). 3) The relationship between P and L viruses is conjectural. On the basis of evidence presented, we suggest as a working hypothesis that the P virus is of the parasitic type, is very small, and spreads by natural means. It is usually masked, and

may be inhibited in tissue extracts by some macromolecular particles. The L virus is larger, has a more limited host range, and may be related to the genome of the leukemic cell.

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Technic for Intravascular Injection and Bleeding of Newborn Rats and Mice.* (24365)

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Recent quantitative studies of immunological tolerance have focused attention on the need for special technics of bleeding and injection of embryonic and neonatal animals. Billingham and Brent(1) found that a higher proportion of tolerant mice was produced when homologous cells were injected by the intravenous route into the newborn than by other routes of administration. Their method of intravenous injection requires considerable skill and is not suitable for bleeding neonatal mice. Terasaki and Cannon(2) have devised an ingenious technic for cross-transfusion of blood in embryonic chicks. An intravascular method of injection for rat embryos of 13 to 17 days was described by Grazer and Clemente(3) but it has a relatively high mortality rate. The intracardiac technic described here for bleeding and injection of newborn rats and mice was accompanied by mortality of less than 5% in over 600 newborn rats, and 40 newborn mice. This technic has been advantageously employed in studies of transplantation immunity(4), and heterologous tumor cell tolerance.

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Methods and materials. The animal is immobilized on an injecting board ventral side up (Fig. 1) with masking tape. It is important to stretch the subject as it is masked down, which helps to bring the heart closer to skin surface. The injecting equipment consists of a mounted Luer Lock Tuberculin syringe connected to a 15-inch length of P 50 polyethylene tubing by means of a plastic tubing adapter (Clay Adams size A). The 15-inch length can be conveniently calibrated for vol-

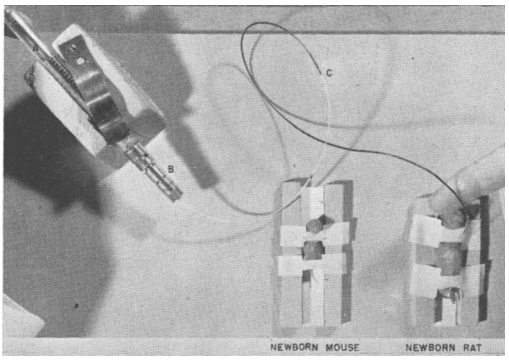

FIG. 1. Injection apparatus and procedure. (A) Luer Lock Tuberculin Syringe. (B) Plastic tubing adapter (Clay Adams Size A). (C) P 50 polyethylene tubing.