TABLE II. Plasma Free Amino Acid Levels (mg/100 ml) Determined by Non-Accelerated and by Accelerated Automated Ion-Exchange Chromatography.

Amino acid	Non-accelerated	Accelerated
Taurine	1.06	.98
Aspartic acid	.10	.04
Threonine	1.79	1.46
Serine	1.04	.85
Asparagine + glutamine	8.65	8.73
Proline	2.34	2.38
Glutamic acid	.84	.78
Citrulline	.38	.37
Glycine	1.59	1.47
Alanine	3.08	3.37
Valine	*	2.15
Cystine	*	1.04
Cystathionine	.04	.04
Methionine	6.20	6.36
Isoleucine	.49	.44
Leucine	1.27	1.19
Tyrosine	.86	.80
β-Alanine	>.00	>.00
Phenylalanine	.72	.68
Lysine	2.96	3.01
Histidine	.92	.83
Arginine	1.51	1.44

* Not resolved by the ion-exchange column.

done. Hamilton(4) compared the levels of a few amino acids in protein-free filtrates from the same serum deproteinated with 3% sulfosalicylic acid and with picric acid and found no significant differences.

Gerritsen *et al*(6), in a more comprehensive study found that 13 of the 19 amino acids and other ninhydrin-reacting substances were higher in pooled serum deproteinated by picric acid than by sulfosalicylic acid.

In their study, serum was deproteinated with 3% sulfosalicylic acid; in the present investigation, plasma deproteinated with 20% sulfosalicylic acid was used. The difference in results between the 2 investigations possibly could be due to these factors.

Summary. A comparative study of 20% sulfosalicylic acid and of 1% picric acid in precipitating plasma proteins before analysis for free amino acids by ion-exchange chromatography showed that both methods give values comparable in precision and accuracy. The sulfosalicylic acid method is less time-consuming, and requires fewer manipulations.

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Studies on a Murine Lymphoma Induced by Reovirus Type 3: Some General Aspects of the Lymphoma 2731/L.* (31334)

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The clinical and histopathological pictures of acute and chronic disease of mice following neonatal infection with types 1, 2 and 3 reovirus have been fully reported(1,2,3). The development of the long-term chronic disease following reovirus type 3 infection of neonatal mice indicates chronic immunological damage and associated premalignant changes(4). One

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result has been the development of a lymphoma(4), the properties of which have been briefly described and the similarities to Burkitt's lymphoma as it occurs in Africa pointed out(5,6).

It is now commonly believed that Burkitt's lymphoma is associated with the mosquito transmission of a virus(7), and, of the agents isolated from human biopsy specimens, reovirus type 3 is perhaps the most convincing and the most frequent(8). We have recently described the isolation of reovirus 3 from mosquitoes in Australia(9) and this observation has since been confirmed by Miles *et al*(10). Reovirus 3 has also been isolated from the blood of birds, cattle and rodents and mosquito transmission of reovirus 3 between vertebrates almost certainly occurs in the Australasian environment(9,10).

This interesting association of reovirus 3 with Burkitt's lymphoma encouraged us to commence detailed study of our murine lymphoma (2731/L) which appears to have been induced as a result of reovirus type 3 infection. We report here on some introductory observations which outline the gross picture of 2731/L.

Materials and methods. Experimental animals: Mice, (1) Prince Henry (PH) strain of albinos(11) were host animals for maintenance and production of 2731/L cells used in these experiments. (2) Royal Perth Hospital (RPH) strain of albinos, origin unknown but maintained as a closed colony for the last 16 years. (3) A Gowan strain of albinos maintained on a random sib mating principle since 1963. (4) NZB/Bl strain (*idem*). (5) A Balb/C strain (*idem*). (6) A C3H strain (*idem*). Rats, (1) A Wistar strain of albinos maintained by random sib matings since 1962. (2) A Buffalo strain (*idem*). Guinea pigs, maintained on a random flock principle and originated from an unknown source.

Preparation of cell-free extracts. 2731/L cells were frozen and thawed 3 times with grinding and made up to a 10% suspension in Hanks' buffered salt solution (HBSS). This suspension was centrifuged at 3000 rpm for 30 minutes on Head 831 of an International Refrigerated Centrifuge. The resulting supernate was designated cell-free extract.

Storage at —70°C. 2731/L cells were dispersed in 10% dimethyl sulphoxide in 199 and 10% fetal bovine serum, as a concentrated cell suspension. This was sealed in 1 ml volumes in glass ampoules and stored at —70°C in a dry ice cabinet. Viability was tested at intervals, by trypan blue staining and inoculation into litters of day-old PH mice.

Virus isolations. Tissues tested for virus isolation were frozen and thawed 3 times and treated as described above for preparation of cell-free extracts. The preparations were given up to 3 blind passages on mouse L-cells and/or primary monkey embryonic kidney cells (rhesus). Standard methods of cell maintenance were used. Tests were considered negative if no cytopathic effect occurred and haemagglutination of the sonicated tube contents were negative with human 'O' and/or bovine red blood cells. Virus was typed by haemagglutination-inhibition (HI) tests using reovirus type-specific serum prepared in PH mice.

Sera. Test sera used for HI activity were heat-treated and Kaolin-treated by standard procedures.

X-irradiation studies. 25 PH mice were given 150 r in 4 weekly doses of 3 minutes 52 seconds duration. Total irradiation time was 15 minutes 28 seconds and radiation-free time within the course was 18 days. The machine used was a Maximar 220 Kv 15 mA (HVL 1.20 mm Cu).

Results. Host range, species, strain and age differences. Stock 2731/L cells were grown in infant PH mice and approximately 10^7 cells in HBSS were injected by the intraperitoneal (IP) route into test animals. The animals were observed daily for development of tumors, kept until moribund and then killed for post-mortem examination. Observations are recorded in Table I.

Macroscopic tumor distribution. It became apparent during initial experiments that the distribution of tumors could be modified to some extent by route of inoculation of 2731/L cells. The results are summarized in Table II.

Microscopic distribution. Histopathological

surveys show that the leukemic cell infiltrates throughout the whole body. This appears to be through overflow of cells from the reticuloendothelial system both before and after the macroscopic preferences have been saturated. Titration of 2731/L cells in PH mice was carried out as IP inoculations into day-old infant mice. The results are detailed in Table III.

Cell-free extract studies. 16 mice were in-

TABLE I. Comparison of Lymphoma Development in Animals 24 Hours Old and + 3 Weeks of Age at Time of Inoculation.

	24 hr old at inoculation				+3 wk old at inoculation			
	No. inoculated	No. with lymphoma	% with lymphoma	Age at autopsy (days)	No. inoculated	No. with lymphoma	% with lymphoma	Age at autopsy (days)
Mice: PH	+2000	+2000	100	8–11	+2000	+2000	100	+28
RPH	32	32	100	8–11	24	0	0	+35
Gowans	16	16	100	8–11	23	0	0	+35
Balb/C	18	18	100	6– 9	40	40	100	35–42
C3H		Not tested			15	0	0	Young adults
NZB/Bl		" "			3	3	100	35
Rats: Wistar	5	2	40	8–12	6	0	0	56
Buffalo	9	7	78	8–12	1	0	0	56
Guinea pigs						Not tested		
Rabbits*	11	0	0	30–40				
		Not tested			6	0	0	?

* Rabbits used in sera preparation studies; therefore cell numbers received far exceeded 10^7/ml.

TABLE II. Distribution of 2731/L with Route of Inoculation in PH Mice (3 Weeks).

Route	Jaw	Thymus	Lungs	Liver	Spleen	Kidney	Mesenteric lymph node	Mesentery	Other lymph nodes	Ovaries	Male genitalia	Subcutaneous	Ascites	Brain	CNS	% no take
Intraper.	1+	1+	—	4+	—	1+	4+	4+	—	1+	1+	—	1+	—	—	<1
Intrav.	1+	—	—	1+	—	4+	4+	4+	—	2+	2+	—	1+	—	—	<1
Subcut.	—	—	—	—	—	—	1+	1+	—	—	—	4+	—	—	—	9
Oral	1+	—	4+	—	—	—	—	—	—	—	—	—	—	—	—	38

\+ Tumor development — No tumor development

TABLE III. Titration of 2731/L in Day-Old Mice.

No. cell/mouse	Days to autopsy	No. with lymphoma	Total No.	% Lymphomas
4×10^5	10–11	8	8	100
4×10^4	11–13	6	6	100
4×10^3	15–17	8	8	100
4×10^2	15–17	5	5	100
4×10^1	18–31	8	8	100
4	23–35	2*	7	29
0.4	28	0†	6	0

* Here, at post-mortem examination, one animal (D35) had a large jaw tumor (submaxillary gland).
† All animals here were retarded in growth and died on days 27-28. No tumors were present but spleens and thymi were atrophied.

oculated with cell-free extract at 24 hours of age; all survived and were kept up to 209 days. Total blood counts were carried out at post-mortem examination and all counts were normal for age. Macroscopically there was no tumor development of any kind.

The cell-free extract was given 2 blind passages on MEK and L cells and at each passage samples were inoculated into day-old mice. In all, 27 mice were inoculated; none died and at post-mortem examination, 140 days later, all were normal with no tumor development or evidence of infective virus. Total white counts were normal.

Storage at —70°C. 2731/L cells were stored for over 250 days at —70°C. Samples of these cells were inoculated into day-old mice and on 7 occasions up to 250 days regrowth occurred involving a progressively decreasing percentage of mice and an increasing time before the macroscopic appearance of the lymphoma. If the lymphoma appears, it does so within 41 days and, to date, no late development of the lymphoma has occurred.

Serum studies. All serum samples taken during the experiments have been routinely tested for HI antibodies to reovirus type 3. All have been negative ($<1/10$).

Virus isolation studies. Routinely throughout these experiments samples have been taken and prepared for virus isolation. All tests have yielded no evidence of infective virus.

Light microscopic measurements. Giemsa-stained impressions of 2731/L have been prepared. The population of cells is divisible into 2 groups, size ranges being nuclear diameters $4.2 \pm 1.0\ \mu$ and $6.5 \pm 0.5\ \mu$ and overall diameters $4.5 \pm 1.0\ \mu$ and $7.3 \pm 1.0\ \mu$. The cells are approximately circular and mainly of lymphoblastic type (Fig. 1).

Associated observations. The oxyhaemaglobin content of blood of animals with 2731/L is often depressed [normal PH oxy-Hb (4 weeks) 14.0 (13.4-15.9) g%; after 2731/L growth 8.1 (6.0-10.2) g%].

Mice bearing the 2731/L tumor have on occasion developed pancreatic obstruction which has yielded a false reovirus type 3 oily hair effect (OHE)(1). Virus isolations have been negative. We frequently obtained runting and lymphoma in the one animal and subcutaneous passages in mice are often associated with splenic atrophy (to be reported elsewhere). Splenic atrophy is common in mice bearing tumors following IP inoculation and the thymus is rarely enlarged.

X-irradiation studies on PH mice. The natural incidence of leukemia in PH mice is extremely low. X-irradiation of PH mice yielded 52% developing thymomas and splenomegaly from between 86 and 151 days.

Discussion. Mice and rats may be considered to be immunologically immature to some isoantigens at 24 hours of life(12) and therefore growth of genetically different invasive cells is to be expected. Table I illustrates that 2731/L is able to establish itself in all 24-hour-old mice and in the majority of rats of all strains tested. 2731/L may also be serially passaged in these strains. In strains other than the PH strain, there is often the

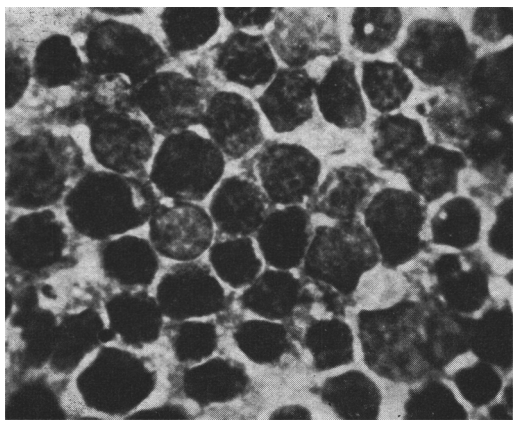

FIG. 1. Giemsa-stained impression of 2731/L cells. (× 840)

early development of a runting syndrome. However, this is not one characterized by the splenomegaly associated with immunological runting but is one associated with thymic and splenic atrophy very similar to that described by Stanley *et al*(1) resulting from reovirus type 3 inoculations at limiting dilutions. In the case of the rat inoculations, severe weight loss occurred over the first 3 days and at least 4 of the rats died with macroscopic appearance of the runting associated with splenic atrophy. Normal immunologically competent PH mouse mesenteric lymph node cells in similar concentrations inoculated into 11 rats 24 hours old gave none of the symptoms described above and the rats were normal at 30 days of age. The 2731/L cells used in these experiments had been routinely maintained as a PH baby line passage; therefore it is unlikely that the tumor was contaminated with normal immunologically competent cells. The results suggest that 2731/L cells may be able to mount an acute immune response of their own, similar to the graft-*versus*-host response(13) but without splenomegaly; or that 2731/L contains reovirus in a form capable of producing only the chronic syndromes of reovirus type 3 infection(2,4). Some accompanying syndromes in the natural host (PH mice) such as runting plus lymphoma, splenic atrophy, and depressed oxy-Hb content all suggest immune responses. Furthermore, all tests for the presence of infective virus have been negative in these situations. Resistance to transplantation experiments to be reported elsewhere have indicated that PH mice cannot themselves be actively immunized against 2731/L cells. Dameshek and Schwartz(14) suggest that the only difference between a response to an antigen and a leukemia plus auto-antibody production is the fact that in the former case cell proliferation decreases when antigen is removed and in the other case cell leukemia results from continued proliferation which is uncontrolled. However, if the "auto-antibody" was, in fact, a product of the leukemia cells against the host, then antigen would not have been removed. The Bence-Jones proteins may be taken as evidence that cells of the leucocyte system can produce antibody-like proteins after they have undergone neoplastic change (15). Therefore it is possible that 2731/L cells are producing "auto-antibody" while they are recognized by the PH host animal as self. Mice growing subcutaneous 2731/L tumors may develop circulating cytotoxic antibody. However, whether this is auto-antibody or pseudo isoantibody is not yet clear.

2731/L could conceivably be an uncontrolled proliferative cell system which is itself causing the splenic and thymic atrophy. Work is proceeding in an attempt to clarify this situation. Animals able to reject the 2731/L cells at 24 hours of age are those considered to be immunologically more mature at birth, for example guinea pigs (Table I). In all cases the young animals dying of 2731/L lymphoma do so within 12 days. This result suggests that there is no inhibition of cell proliferation with different hosts and that the young mouse is, in fact, only a biophase providing optimal conditions for cell proliferation.

Attempts to grow 2731/L cells in immunologically mature animals of various strains produced for the most part expected results (Table I). 2731/L was rejected and destroyed in most cases. The NZB/Bl strain succumbed to the lymphoma and although they are known to suffer from autoimmune disease, this would not be expected to interfere with normal immune responses to isoantigens at 5 weeks of age. The results suggest that their immune response is generally impaired or that the NZB/Bl strain shares one or more histocompatible antigens with the PH strain.

The Balb/C strain behaved similarly (Table I). PH competent cells were inoculated into day-old Balb/C and vice versa and classical immunological runting was produced. Therefore it was expected that the Balb/C strain should have mounted an immune response to 2731/L. The origin of the PH strain of mice is unknown. However, if the strain originated from a Balb/C strain then even though immunological runting could be produced, major histocompatible antigens may be the same and thus allow the very aggressive 2731/L cell to grow up to give the result of 40/40 dying

from the lymphoma. Chromosome analysis of 2731/L to be reported in detail elsewhere yields a diploid range with a slight shift to 41 (normal PH mode is 40). Hauschka(16) has reported that leukemias with modal values of the type of 2731/L have restricted host ranges. Therefore with the above two exceptions this seems to be so.

Goldie *et al*(17) as long ago as 1955 showed that the route of inoculation could influence pattern of tumor development. The route of inoculation of 2731/L into its "natural" host, the PH mouse, seems to determine the distribution of tumors (Table II). IP and IV inoculation yield distributions, in young adults, which suggest that these routes eventually lead to the same route of transfer of cells throughout the body, this being the reticuloendothelial system. There are subtle differences, however, in that IV inoculation gives higher incidences of kidney and, in the case of females, ovarian development. Also jaw development seems to occur more readily. These distributions are markedly similar to that of Burkitt's lymphoma (18). However, the jaw development could be due to trapping of the cells in the submaxillary gland and not due to outgrowth through the jaw bone from the bone marrow, which is characteristic of Burkitt's lymphoma (19). It may therefore be that the similarity to Burkitt's lymphoma in distribution is due to that of a common route of spread of cells through the body. Oral inoculation yields tumor development restricted to the jaw and lungs. This seems to indicate that the tumor cell can be trapped in the mucosa and infiltrate through into surrounding tissue. Tests on distribution of tumors with sex and age have shown that age has no effect on the almost 100% take with PH strain and that in females the ovaries are susceptible at all ages while in males the seminal vesicles are likely to be involved. Relationship to Burkitt's lymphoma in distribution therefore seems to be incidental and any such discussions may, in fact, be on laboratory artifacts only. However, splenic atrophy is often associated with Burkitt's lymphoma (personal communication) and the peripheral lymph nodes and thymus are rarely involved. This is also the case with 2731/L although histopathological studies show that the 2731/L cell may be present in these organs.

Möller(20) has shown that specifically "immunized lymphoid cells" have a characteristic cell-bound cytotoxic effect which is apparently mediated in the absence of antibody. Granger and Weiser(21) report similar results with immune macrophages. However, the results are not conclusive. Both methods are relatively crude and low levels of antibody may not be detected. Gowans (22) suggests a cell-bound antibody functions in host-*versus*-graft rejections. This antibody may not require complement for its cytocidal effect(23). An antibody of this type would not be detected by the methods of Möller and Granger and Weiser. Immunological typing of this kind may occur within the peripheral lymph nodes which restricts tumor spread in these areas. This would suggest that the normal PH mouse does recognize 2731/L as foreign antigen in some way. It has yet to be determined whether this could be due to a new cellular antigen which may or may not be viral specific.

Titration of 2731/L cells (Table III) has shown that the lymphoma is truly cell-bound and is highly reproducible. This is further supported by the storage characteristics, rapid regrowth and finite life.

Cell-free extracts have not produced leukemia. Classical methods of preparing cell-free extracts by freeze-thawing techniques have proved negative as did the method outlined by Lieberman and Kaplan(24). The passage of cell extracts over cells *in vitro* did not increase the potency or decrease the time to tumour formation by a leukemia virus. To date, therefore, attempts at isolation of a leukemia virus have proved negative. X-irradiation studies indicate that a latent leukemia virus is probably present in the PH stock(25). Attempts to implicate this with 2731/L have proved unsuccessful. Papadimitriou(6) has produced electron micrographic evidence for "virus-like" particles present in 2731/L. He suggests that these may be artifacts. Attempts to demonstrate complete infective reovirus particles in 2731/L cells have been unsuccessful. All sera collected throughout these ex-

periments have been tested for HI antibodies to reovirus type 3 and have proved negative ($<1/10$). Therefore if reovirus is still present in the 2731/L cells it is in an occult form protected from normal induction and antibody response. Preliminary trials in attempts to "induce" reovirus type 3 from 2731/L have met with limited success and will be reported in detail elsewhere.

Cell size and morphology studies based on methyl alcohol-fixed, Giemsa-stained preparations (Fig. 1) indicate that 2731/L is made up of lymphoblastic cells, the dimensions of which compare favorably with those arrived at *via* electron microscope measurements(6).

The possibility remains that 2731/L is in fact a spontaneous leukemia. However, one could postulate a case of chronic immunological injury, mediated by reovirus type 3 and manifested as runting(2,4), where autoantibodies and/or cell mediated cytotoxicity are produced. This finally escalates into leukemia(4,5). A similar situation has been proposed by Schwartz and Beldotti(26). The demonstration of virus, or viral antigen, while lending strong support, would in no way prove that 2731/L was in fact initiated directly by reovirus infection. The whole system(1,2) is initially saturated by reovirus type 3 and demonstration of virus may only be in support of the initial environment. However, there is much support from the field of oncogenic viruses(27) that cell transformation by viruses, to the neoplastic state, leaves behind, if not infectious particles, a viral specific antigen which can persist through many transplant generations(28). Therefore, the demonstration of "auto-antibodies," cell mediated cytotoxicity and reovirus type 3 antigen in some form, either by induction of virus or by immunological or serological means, would clarify any relationship between 2731/L and a spontaneous or experimental origin.

Summary. Preliminary studies on a murine lymphoma induced by neonatal infection with reovirus type 3 are reported. In the mature animal, the lymphoma has a restricted host range while in the neonate such restriction does not operate. The development of lymphoma is associated with the intact primitive living lymphoblastic cell which has a definite storage time. All attempts to isolate a murine leukemia virus have proved unsuccessful as have attempts to locate complete infectious reovirus type 3. The organ distribution of the lymphoma and some associated immunological features are discussed with consideration of a possible relationship to Burkitt's African lymphoma.

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Immune Globulin Levels in Colostrum and Breast Milk, and Serum From Fomula- and Breast-Fed Newborns. (31335)

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Breast milk, particularly colostrum, has significant quantities of antibody-containing immune globulins. The antibodies secreted by the breast are chiefly in the γA globulin and parallel the elevated levels of γA globulin in colostrum or breast milk(1).

Studies indicating absorption of intact proteins(2) and antibodies(3) from the gastrointestinal tract led us to reinvestigate whether colostral or breast milk immune globulins, particularly γA, are absorbed intact to any significant degree. We, therefore, compared the serum concentration of γG, γM, γA of breast-fed and formula-fed infants and determined the concentration of immune globulins in colostrum and breast milk.

Methods. Cord serum was obtained from 10 full-term infants who were subsequently breast-fed and from 10 full-term infants given formula feedings. A second serum sample was obtained at 4 days from each infant. During this period the mean weights of the breast-fed infants fell from 3280 to 3160 g and of the formula-fed infants from 3100 to 3060 g. Samples of breast milk from the mothers of these infants, obtained during the first 24 hours after delivery and for 3 days thereafter, were frozen immediately and stored at −10°C.

Immune globulin levels were measured quantitatively by the radial diffusion technic of Mancini and associates(4) with specific antiserum to γG, γM and γA globulin incorporated into agar plates. Purified immune globulins quantified by Kjeldahl nitrogen analysis were used as standards. In this laboratory, the method has an error of 3% when samples are tested in duplicate on the same plate or 10% when duplicate samples are tested on different plates. In adults, the normal values by this method(5) are as follows:

γG	1158 mg/100 ml	± 305
γM	99 mg/100 ml	± 27
γA	200 mg/100 ml	± 61

The student T test was used for statistical comparison.

Results. Fig. 1 illustrates the changes in immune globulin concentration in breast secretions during the first 4 days of transition from colostrum to mature breast milk.

The mean values (ml/100 ml) for initial colostrum (γG 43, γM 159 and γA 1735) compared to fourth-day post-partum breast milk (γG 4, γM 10 and γA 100) indicate a high content of γA in colostrum and a small amount of γG globulin.

The immune globulin levels of formula-fed and breast-fed infants were not significantly different (Table I) nor were there significant differences between cord and 4-day serum. Only 2 infants demonstrated detectable γA in cord and 4-day serum.

Assuming a 25% intravascular distribution (6) and a plasma volume of 50 ml/kg, absorption of 60 mg γA globulin from the gas-

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