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MALIGNANT CHANGES IN NEW ZEALAND BLACK MICE

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SUMMARY

Ageing, Coombs positive, NZB mice, may spontaneously develop a neoplasia of the reticulum cell type which can be transferred by serial passage of their lymphoid tissues in young intact or neonatally thymectomized syngeneic recipients. The recipients (103/117) of cell suspensions prepared from the enlarged spleens and/or lymph nodes of four such donors developed an extensive and lethal reticulum cell neoplasia affecting the spleen, lymph nodes, lungs and liver but the bone marrow, thymus and kidneys were seldom involved. The recipients (16/17) of spleen cells from a fifth donor showed massive proliferations of eosinophils in all the organs examined.

Prematurely positive antiglobulin (Coombs) reactions were detected in only two recipients. Although there was an indication that the IgM content of the sera decreased as one of the passages progressed, the levels of IgG and IgA were not seriously distorted.

Particles resembling murine leukaemia virus were identified by electron microscopy in the spleen and in plasma or serum pellets of passage recipients. However, similar particles were also seen in the thymus and/or spleen or bone marrow of untreated NZB mice including an 18-day embryo and animals aged 1–56 weeks, although no particles were found in plasma and serum pellets of mice aged 6–70 weeks.

The theory that autoimmunity, malignancy and virus infection are directly related is discussed.

INTRODUCTION

Mice of the New Zealand Black (NZB) strain, which spontaneously develop haemolytic anaemia (Bielschowsky, Helyer & Howie, 1959; Helyer & Howie, 1963) positive direct and indirect antiglobulin (Coombs) reactions (Holmes & Burnet, 1963; Long, Holmes &

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Burnet, 1963) and have germinal centres in their thymic medulla (Burnet & Holmes, 1964), are being used increasingly to study autoimmune phenomena. We have also reported that the sera of these mice contain large amounts of 19S macroglobulin and have described two histological patterns shown by their lymphoid organs (East, de Sousa & Parrott, 1965). The first phase, seen in animals of 3–11 months, was one of lymphoid hyperplasia in the white pulp of the spleen and the cortex of the lymph nodes, and a small proportion of mice in this age group had thymic lymphomas which were transplantable. The second phase was often characterized by massive proliferations of plasma and/or reticulum cells in the red pulp of the spleen and the medulla of the lymph nodes which caused the gross splenomegaly and lymphadenopathy typical of NZB mice older than 11 months. This proliferation was so extensive as to suggest neoplastic transformation and prompted the experiments described below.

Cell suspensions of the spleens and lymph nodes from old Coombs positive NZB donors were passaged serially through young intact or thymectomized NZB recipients. Organs of both donors and recipients were examined histologically and serologic studies were made of their sera. Electron microscopy was employed to investigate the possible implication of a transmissible agent. A short preliminary comment on these experiments has been included in a previous publication (East & de Sousa, 1966).

MATERIALS AND METHODS

Animals

Our colony or NZB mice was established in November 1963 from eleven pairs of animals supplied by the M.R.C. Laboratory Animals Centre, Carshalton, England. These were F_1 progeny of stock received directly from Dr F. Bielschowsky, University of Otago, New Zealand, which had been inbred for fifty-three generations. The strain has now been maintained by brother–sister matings for a further six generations. The mice were given normal pelleted diet and water *ad libitum* but were isolated from other strains.

The donors were nine old, sick, Coombs positive mice, taken at random from the colony and designated A-I. Most recipients were intact newborn or young adult animals but some were thymectomized 15–63 hr after birth using anaesthesia produced by cooling (East & Parrott, 1962). Each series of passages was identified by the designation of the original donor.

Cell suspensions

The spleens or lymph nodes were removed from the Coombs positive donors under sterile conditions, minced coarsely with scissors in sterile isotonic saline or phosphate-buffered saline (pH 7·2), and the suspensions injected intraperitoneally in a volume of 0·05–0·1 ml containing approximately $60–100 \times 10^6$ cells.

Spleen cells from young, healthy, Coombs negative, NZB donors aged 11–27 weeks, were used as control material and were injected into comparable NZB recipients in doses equivalent to $8·5–60 \times 10^6$ cells.

Direct antiglobulin (Coombs) test

Mice were bled from the tail into heparin and the method of estimation has been described previously (East *et al.*, 1965). The antiserum, rabbit anti-mouse globulin (Microbiological

Associates, U.S.A.), was used in dilutions of 1:5, 1:25, 1:125 and the intensity of the reactions graded from − or ± to ++++.

Sera and plasma

Serum separated from blood withdrawn from the axillae of lightly anaesthetized mice was used immediately or stored at −20°C. Plasma from heparinized blood was always used fresh.

Histology

Sections of thymus, spleen, mesenteric, and axillary and inguinal lymph nodes, lungs, liver and kidneys were cut routinely at 3 μ, and stained with haematoxylin and eosin, methyl green–pyronin (MGP), or periodic acid–Schiff (PAS). The mediastinal region of thymectomized mice was also examined. Imprints of spleen, lymph nodes, bone marrow and smears of tail blood, were stained with May–Grünwald–Giemsa.

Electron microscopy

Samples of sera or plasma were diluted to 10 ml with sterile phosphate-buffered saline (pH 7·2) or distilled water, centrifuged at 1650 *g* for 10 min, and the supernatants centrifuged for one further hour at 105,000 *g* in a Spinco model L centrifuge. The resultant pellets were fixed in 1% Millonig's buffered osmium tetroxide (Millonig, 1961) at 4°C for 1 hr, then dehydrated in graded alcohols and stained for 1 hr in a 0·5% solution of uranyl nitrate in methacrylate, and embedded in methacrylate. Small pieces of spleen and thymus, and fragments of bone marrow were also fixed in 1% osmium tetroxide and prepared as above. Sections were cut on a Porter Blum microtome and examined in a Siemens Elmiskop 1 at 80 kV. Usually 2–3 grids of sections were taken from each block and a maximum of ten blocks from each organ examined.

Serological techniques

Stored sera from individual NZB donors, NZB recipients and untreated NZB controls of a comparable age, as well as samples of pooled sera from normal adult C3H/He mice, were examined by immunoelectrophoretic analysis (Scheidegger, 1955; Wieme, 1959). They were tested and stained in batches of ten on a single glass photographic plate. The rabbit anti-mouse antiserum used, R13/65, was specific for IgM and IgG.

In order to obtain an approximate quantitative assessment of IgM and IgA, two-fold dilutions (1:1 to 1:32) of each serum sample were placed in circumferential wells and examined by the agar-gel diffusion technique of Ouchterlony against appropriate antisera, using a modification of the method described by Gell (1957). The titre was estimated as that dilution at which the precipitin line approached most closely to the antigen well. Specific antisera for IgA (G105Ā) and for IgM (R113Ā), both provided by Dr J. L. Fahey (Fahey, Wunderlich & Mishell, 1964), were used.

RESULTS

Cell suspensions prepared from the enlarged spleens or lymph nodes of five NZB donors, designated A–E and aged 47–76 weeks, were passed serially by intraperitoneal injection in a total of 134 intact or neonatally thymectomized NZB recipients (Tables 1 and 2).

Post-mortem findings

The first recipients of donor material in all the series developed, within 49–185 days of injection, very much larger spleens than untreated mice of the same age. Some of these recipients in Series A, B, D and E survived for a further 68–211 days despite having spleens

TABLE 1. Serial passage of cell suspensions of the lymphoid organs of old NZB donors in young intact NZB recipients

Series	Pass No.	Cell suspension	Age of recipients (days)	No. mice with splenomegaly/ No. mice injected	Latent period (days)
A: Donor female, age 50 weeks	1	(a) Spleen	1	5/5	103*–334
	2	(a) Spleen	21	0/2	—
			76	1/2	96
	3	(a) Spleen	1	5/5	30–32
	4	(a) Spleen	2	2/4	66, 70
	5	(a) Spleen	5	5/5	27–33
	6	(a) Spleen	5	4/4	27–28
			15	0/4	—
B: Donor female, age 57 weeks	1	(a) Spleen + lymph node	1	5/5	156–396
	2	(a) Spleen	4	3/3	51–65
		(b) Lymph node	4	2/2	62, 72
	3	(a) Spleen	2	2/2	45, 48
		(b) Lymph node	6	6/6	35–90
	4	(a) Spleen	2	4/4	33–35
		(b) Lymph node	11	3/3	44–50
	5	(a) Spleen	11	4/4	38–41
		(b) Spleen	9	1/1	28
			6	2/2	28, 28
	6	(a) Spleen	6	5/5	38–67

* Mouse killed moribund with enlarged spleen as donor for subsequent passage.

that could easily be palpated. However, by the third pass all the recipients in Series A–D became very ill only 18–90 days after injection and had massive spleens which weighed 1–3 g post-mortem (controls 120–180 mg). The condition of the lymph nodes varied from series to series but was reasonably consistent throughout each series. In one series (B) all the nodes were very large and haemorrhagic, in another (C) the mesenteric node was mainly affected, while in Series A all the nodes were of normal size although the inguinals were haemorrhagic. In animals of Series A–D the liver was thickened and pitted or nodular,

the lungs were pale and spongy, but the kidneys were normal. On occasion, the thymus was slightly enlarged but, usually, it was normal in size and colour.

Although large spleens and lymph nodes were also seen in the first recipients of Series E, these organs were only slightly enlarged in the thymectomized and the intact recipients of the second and third passes respectively. The general condition of the mice comprising

TABLE 2. Serial passage of cell suspensions of the lymphoid organs of old NZB donors in young thymectomized and intact NZB recipients

Series	Pass No.	Cell suspension	Type and age of recipients	No. mice with splenomegaly/ No. mice injected	Latent period (days)
C: Donor female, age 76 weeks	1	(a) Spleen	Thymectomized—18 days	2/2	49†, 69
		(b) Lymph node	Thymectomized—18 days	2/2	63†, 85
	2	(a) Spleen	Thymectomized—7 days	4/4	25–38
		(b) Spleen	Intact—4 days	5/5	21–95
	3	(a) Spleen	Thymectomized—10 days	4/4	18–25
		(b) Spleen	Intact—3 days	1/2‡	28
	4	(a) Spleen	Intact—13 days	0/2	—
		(b) Spleen	Intact—6 days	3/3	21–22
	5	(a) —	—	—	—
		(b) Spleen	Intact—4 days	5/5	15–19
	6	(a) —	—	—	—
		(b) Spleen	Intact—4 days	3/3	14–15
			Intact—10 days	0/2	—
D: Donor female, age 56 weeks	1	(a) Spleen	Intact—1 day	4/4	185†–310
	2	(a) Spleen	Thymectomized—2 days	2/2	42, 113
	3	(a) Spleen	Intact—4 days	4/4	49–72
	4	(a) Spleen	Intact—1 day	5/5	70–101
E: Donor male, age 47 weeks	1	(a) Spleen	Intact—26 days	6/7	139*–253
	2	(a) Spleen	Thymectomized—15 days	3/3	32–43
	3	(a) Spleen	Intact—6 days	7/7	20–50

* Mouse killed moribund with enlarged spleen as donor for subsequent passage.
† Mouse killed healthy with enlarged spleen as donor for subsequent passage.
‡ Received spleen residue after filtration.

pass three was always poor and they had thin, fine, ruffled fur which gradually disappeared from the ventral surfaces. Blood-stained ascitic fluid was found in these mice post-mortem and the thymus was of normal size but yellow in colour. The liver was pale but not enlarged, the lungs pale pinkish-grey, but the kidneys were normal.

Both spleen and lymph node suspensions were effective passage material. The short

latent period in pass one Series C (Table 2) indicated that neonatal thymectomy of the recipients facilitated passage but this point could not be proved under the existing experimental conditions. It should also be noted that the intervention of lethal wasting and the possible activation of the latent hepatotrophic virus, MHV-1, in neonatally thymectomized mice (East *et al.*, 1963) can limit the usefulness of this manœuvre.

In four other experiments, transfer of bone marrow, ascites fluid or spleen tissue from two female and two male Coombs positive donors designated F–I and aged 37–68 weeks, to twenty-three intact recipients was unsuccessful.

Spleen cells from healthy, Coombs negative donors, aged 11–27 weeks did not affect twenty-two control recipients which are now 9 months old.

Direct anti-globulin (Coombs) tests

A total of ninety-nine tests were performed on tail blood obtained from forty-nine recipient mice. These included animals in the successful Series A–E inclusive and from two, and sometimes three, passes within each series. The mice were first tested 13–50 days after injection and thereafter at monthly intervals. They were classed as positive if their tests scored + or more in at least two of the three dilutions of antiserum used. Only two recipients, one intact male of pass three Series A and one thymectomized female of pass one (a) Series C, had weakly positive, premature, reactions 24 and 42 days after injection when they were, respectively, 25 and 60 days old. Of the nine recipients which survived beyond the age at which untreated intact NZB mice begin to develop positive Coombs tests, namely 140 days (East *et al.*, 1965), seven gave weak reactions at the expected time. Of the twenty-three intact recipients used in the four unsuccessful transfer experiments (Series F–I), eighteen were tested on forty-eight occasions. All were negative 8–34 days after passage but subsequently became positive at the same age as untreated controls.

Histological findings—Donors A–E

Each of the five donors showed the pathological changes already described for ageing NZB mice (East *et al.*, 1965) but they differed in the extent to which these changes occurred.

Reticulum cells dominated the enlarged spleens of donors A, B and C, but in donors D and E the red pulp was actively haemopoietic and follicles, with or without germinal centres, were present in the white pulp. Sheets of reticulum cells also replaced the cortex and medulla of the superficial and mesenteric lymph nodes of donor B although they were interspersed with mast cells, plasma cells and PAS-positive globules (East *et al.*, 1965) in donors A, C and E, but lymphoid follicles were not seen. Two types of cells appeared consistently in impression smears of the spleens and lymph nodes. One type, often large and bizarre in shape, had a nucleus of diffuse chromatin which stained pink with May–Grünwald–Giemsa. The cytoplasm was slightly basophilic (Fig. 1). The second type was smaller, with an oval or circular nucleus of irregularly-clumped chromatin which stained a deep red. The finely-granular blue-grey cytoplasm was sometimes defined by a darker basophilic rim (Fig. 3). We do not feel able to classify these cells as other than primitive reticulum cells. The thymus of donor B showed a patch of hyperplastic reticulum cells, germinal centres were found in the medulla of donor A, and the thymus of donor E was normal.

Emboli of reticulum cells also occurred in the sinusoids of the liver and the veins of the

lungs, or invaded the portal and peribronchial spaces of donors B and C, respectively, while the more typical nests of pyroninophilic cells (East *et al.*, 1965) were evident in these same organs of the other three donors. All the donors showed the severe kidney lesions described previously (Helyer & Howie, 1961, 1963; Holmes & Burnet, 1963; East *et al.*, 1965; Mellors, 1965) namely, thickening and hyalinization of the glomerular basement membrane.

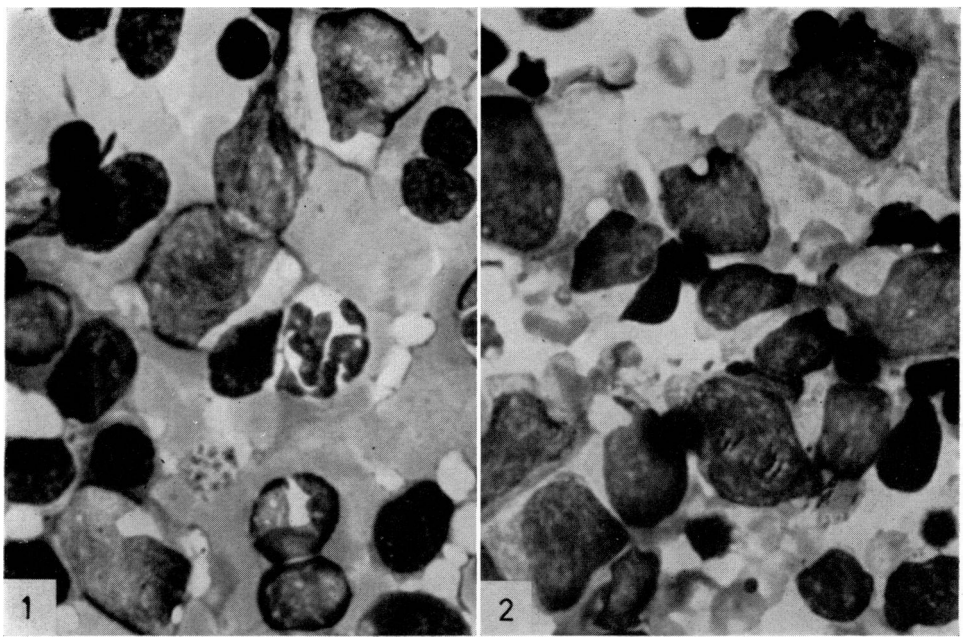

FIG. 1. Impression smear of spleen from the intact female NZB donor of Series A aged 50 weeks showing large reticulum cells containing pale-staining nuclei with delicate diffuse chromatin. May–Grünwald–Giemsa, × 1080.

FIG. 2. Impression smear of spleen from an intact female NZB mouse of Series A which received pass No. 1 spleen suspension from the donor shown in Fig. 1, and which had splenomegaly but normal nodes when killed 200 days after injection at 1 day. Note reticulum cells similar to those shown in Fig. 1. May–Grünwald–Giemsa, × 1080.

Histological findings—Recipients

Histological material from sixty recipients of passes Nos. 1–3 inclusive in Series A–E was examined. All the mice in Series A–D showed a consistent and characteristic reticulum cell neoplasia affecting the spleen, lymph nodes, lungs and liver but seldom involving the kidneys and bone marrow, or the thymus of intact animals.

Pathological changes in the spleen were already well advanced by the first passage although some recipients in Series A still had strands of haemopoietic tissue in the regions corresponding to the red pulp and lymphocytes in the areas normally occupied by the follicles, while the spleens of Series D were primarily haemopoietic. After further passages

the structure of the spleen was virtually obliterated by pale-staining reticulum cells. PAS-positive globules were distributed throughout the splenic tissue except in recipients of Series C.

The superficial lymph nodes, and the mesenteric nodes of most mice except those in Series D, showed the same intense reticulum cell proliferation as the spleen and in the thymectomized recipients, particularly of Series C, they presented a very striking picture.

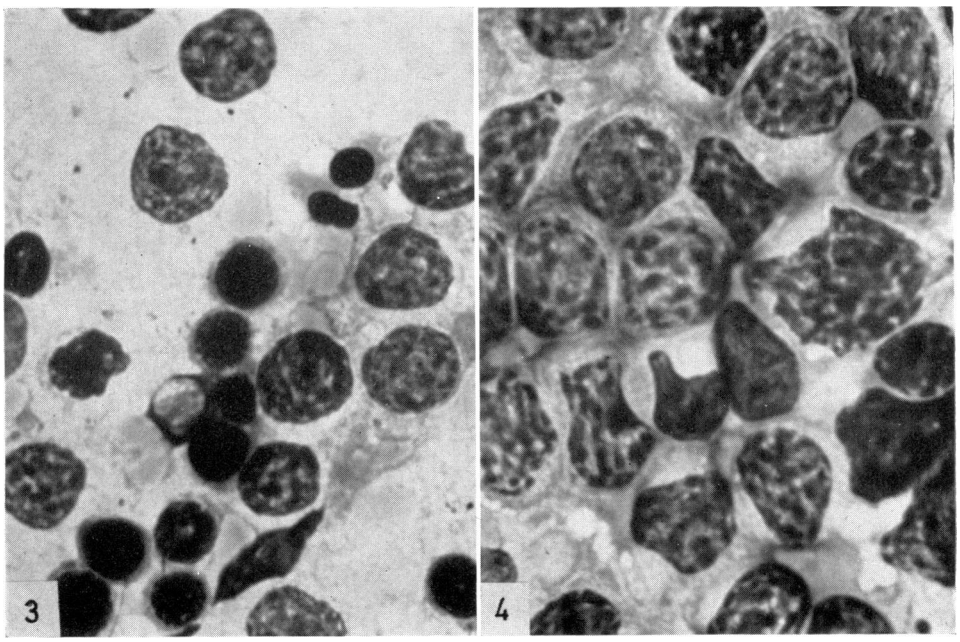

FIG. 3. Impression smear of spleen from the intact female NZB donor of Series B aged 57 weeks. Note that the predominant cell type is of medium size with a large nucleus of clumped chromatin. May–Grünwald–Giemsa, × 1080.

FIG. 4. Impression smear of spleen from an intact female NZB mouse of Series B which received pass No. 4 lymph node suspension at 11 days and which had splenomegaly and lymphadenopathy when it was killed 44 days later. The dominant cell is larger than that illustrated in Fig. 3 but it has the same nuclear pattern. May–Grünwald–Giemsa, × 1080.

Regions of the mid- and deep cortex were apparently exempt from the reticulum cell proliferation (Fig. 6) and it is these areas which, in neonatally thymectomized mice of other strains, are specifically depleted of lymphocytes and have been termed 'thymus-dependent areas' (Parrott, de Sousa & East, 1966). Lesions attributable to the hepatotrophic virus MHV-1 (East *et al.*, 1963) were also seen in the lymph nodes of the thymectomized NZB recipients.

Impression smears of the spleens and lymph nodes of mice in Series A (Fig. 2) and B (Fig. 4) were dominated by primitive cells very similar to those identified in the smears of their respective original donors, although those of Series B were rather larger in size. The

dominant cell type of Series C resembled that of Series A while both 'A'-and 'B'-type cells were present in Series D, with the former in the majority. The lungs and livers of all recipients were also heavily infiltrated with reticulum cells (Fig. 5).

By comparison, the thymus of intact recipients was relatively normal but there were a few small medullary nodules of reticulum cells in some mice in Series B and D. The kidneys, too, remained uninvolved despite the presence of large numbers of reticulum cells in the cuffs of connective tissue surrounding the adrenals. Recipients which survived for a

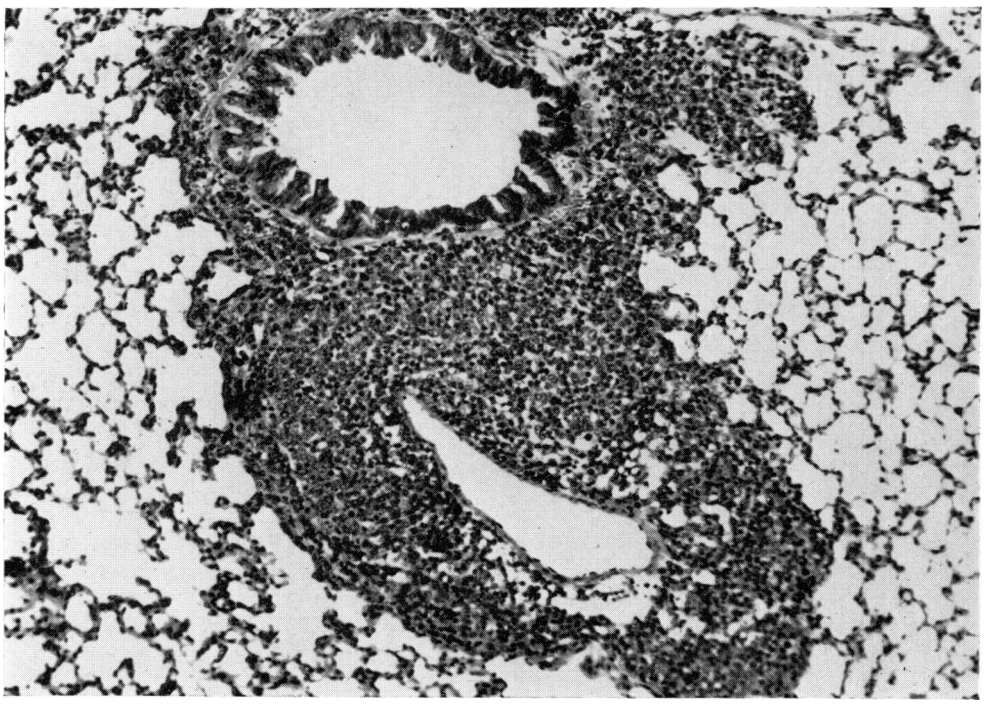

FIG. 5. Lung from an intact female NZB mouse of Series B which received pass No. 3 lymph node suspension at 6 days and which had splenomegaly and lymphadenopathy when it was killed 35 days later. Note the reticulum cells infiltrating the peribronchial space. H & E, × 144.

relatively long period of time had the glomerular hyalinization seen in untreated NZB mice of a comparable age. The bone marrow contained normal proportions of erythroid, myeloid and lymphoid cells, and the blood smears were unexceptional.

The histological patterns which developed in Series E defined it sharply from the others. It was also the only successful series in which intact weanlings instead of newborn recipients were used in the first passage, but this may have little significance since the latent period was not prolonged (Table 2). The enormously enlarged spleens of these weanlings were mainly haemopoietic but their lymph nodes, thymus, kidneys and lungs were comparable to those of untreated controls. However, eosinophils and not reticulum cells became the exclusive cellular component of the lymph nodes, liver and lungs of the thymectomized

recipients of the second passage. They penetrated the thymus-dependent areas of the nodes and also obliterated the bone marrow. By the third passage, the eosinophils were distributed throughout the entire spleen, they infiltrated the bone marrow, lungs and liver, completely replaced the normal thymic architecture and pervaded the blood smears but avoided the kidneys.

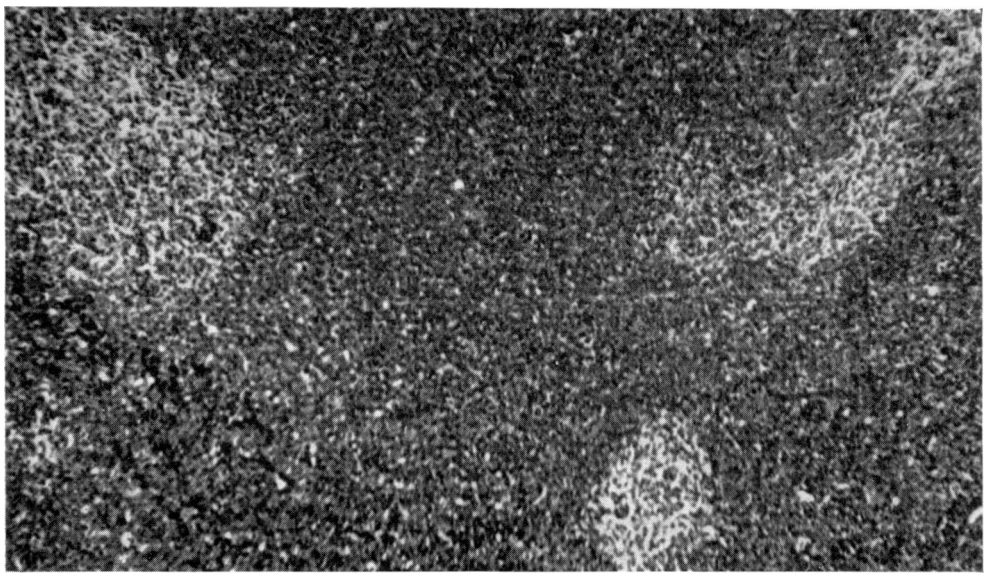

FIG. 6. Inguinal lymph node from a neonatally thymectomized male NZB mouse of Series C which received pass No. 3 spleen suspension at 10 days and which had splenomegaly when it was killed 18 days later. The pale cortical areas, which probably correspond to the thymus-dependent areas, are surrounded by proliferating reticulum cells. H & E, ×108.

Unsuccessful passes

We attribute the failure of the Series F, G and H to the material chosen for the original passage. In two cases, suspensions of bone-marrow cells and in the third case, ascites fluid were injected, but these inocula contained few reticulum cells. The fact that the first recipients of Series I were 23 days old may have affected the outcome of this passage since some reticulum cells were present in the spleen suspension injected.

Electron microscopy

Material from nine recipient and ten untreated NZB mice and from two normal C3H/Bi and two normal C57BL mice was examined.

Recipient NZB mice. Particles resembling murine leukaemia virus were found in varying numbers in serum or plasma pellets of five intact NZB recipients of passes three and four in Series B, C and D which were killed moribund at 4–9 weeks, 21–55 days after injection. An electron micrograph (Fig. 7) shows a serum pellet which consisted almost entirely of such particles—the tails are regarded as artefacts caused by diluting the serum sample with

water. Similar particles, often budding from the surface of lymphoid cells (Fig. 8), were seen in spleen tissue obtained from three of these recipients and from a further four mice killed 35–49 days after the third passage in Series B and D. The mean diameter of 100 such particles was 103 mμ (80–128 mμ) and they consisted of an external unit membrane and a dense central nucleoid. Intracytoplasmic doughnut-shaped particles with double membranes but without the dense nucleoid were the only type seen in one of the spleens from Series D but their significance is unknown.

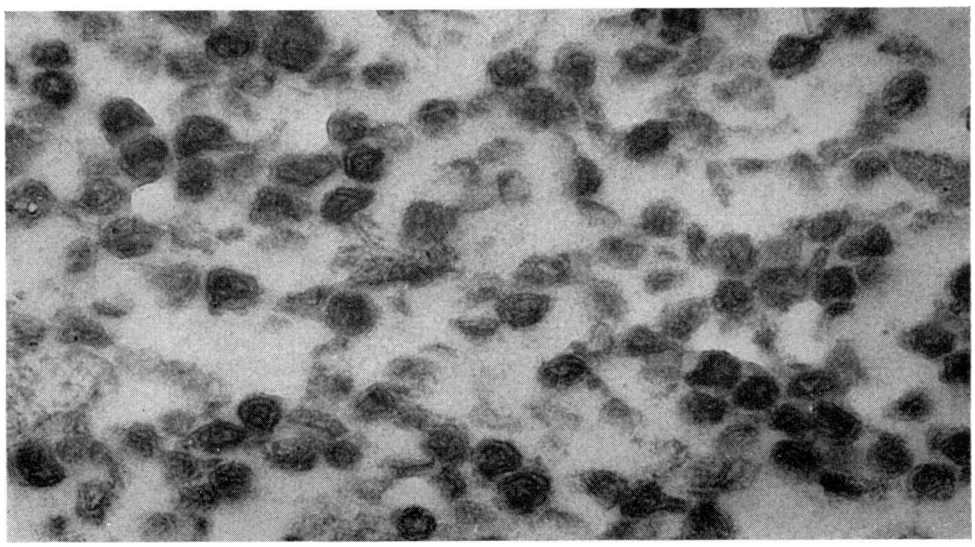

FIG. 7. Virus-like particles in a pellet prepared from the serum of an intact male mouse of Series B which received pass No. 3 lymph node suspension at 6 days, and which had splenomegaly and lymphadenopathy when it was killed 41 days later. × 80,000.

Untreated NZB mice

Particles were also found in thymus tissue of untreated NZB mice including an 18-day embryo and three animals aged 1, 10 and 56 weeks. They were identified in the spleens of the mice aged 1 and 10 weeks but not in those of either the embryo or the oldest animal examined. The mean diameter of 100 of these particles was 109 mμ (80–131 mμ) and, in the spleen of the 10-week-old mouse, they were present in very large numbers. Bone marrow collected by aspiration of the femurs of the mice aged 10 and 56 weeks contained a few particles. However, particles were not identified in plasma or serum pellets of the 10- and 56-week-old mice or in pellets of an additional three young untreated animals aged 6–10 weeks and three old untreated mice aged 57–70 weeks (including the donor of Series B).

Normal C3H/Bi and C57BL mice. No virus-like particles were seen in plasma pellets of two C3H/Bi mice aged 8 and 56 weeks and two C57BL mice aged 7 and 36 weeks.

A pellet from one recipient NZB mouse, and most of the pellets from the untreated NZB, C3H/Bi and C57BL mice, contained inclusions resembling mycoplasma and bacteria.

Serological data

The immunoelectrophoretic patterns of sera from intact NZB donors and recipients of Series A and B and untreated NZB and C3H/He controls, obtained with an antiserum specific for IgM and IgG, are shown in Fig. 9. The quantitative assessment of their constituent immunoglobulins IgM and IgA, using the Ouchterlony technique, is summarized in Table 3.

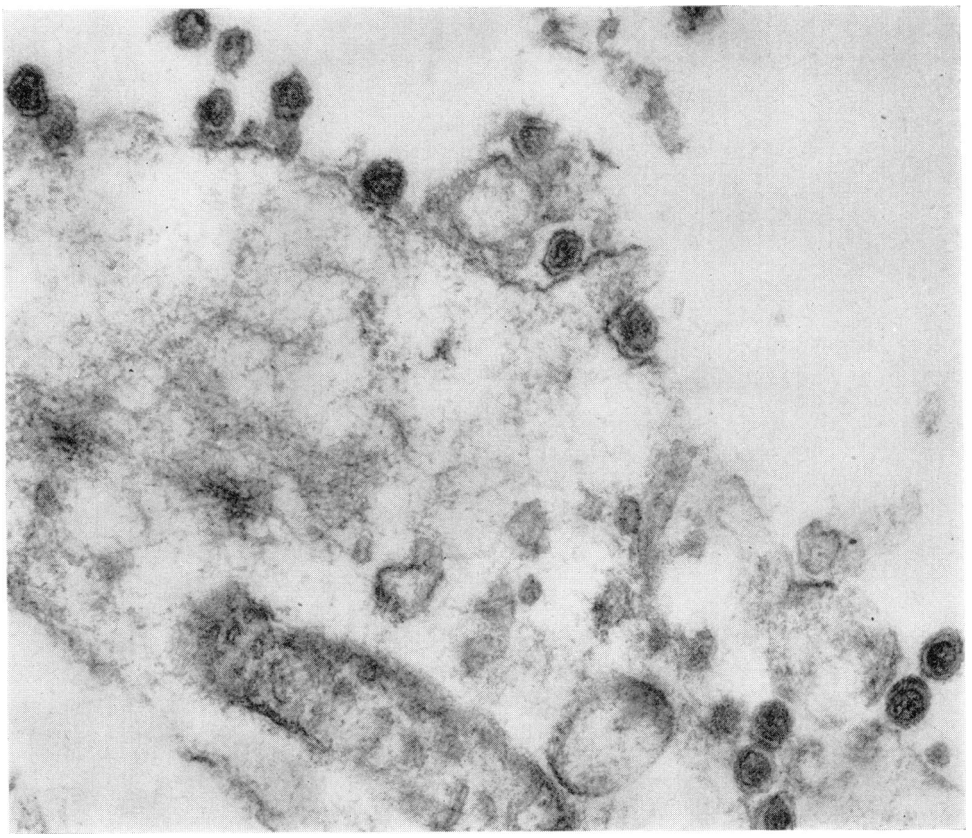

FIG. 8. Virus-like particles budding from the surface of a lymphoid cell in the spleen of an intact male mouse of Series B which received pass No. 3 lymph node suspension at 6 days and which had splenomegaly and lymphadenopathy when it was killed 35 days later. × 70,000.

The amount of IgM present in the sera of the recipients of Series A (sera 3, 5, 7 and 9) did not differ consistently from that of their untreated controls (sera 4, 6 and 8), and all the NZB mice had as much, and usually more, macroglobulin than untreated animals of the C3H/He strain (sera 1 and 10). However, by comparison with their untreated controls (sera 14, 17 and 19), the IgM content of the recipients of Series B (sera 13, 15, 16 and 18) appeared to decrease as the passages progressed and was often smaller than that of the C3H/He mice

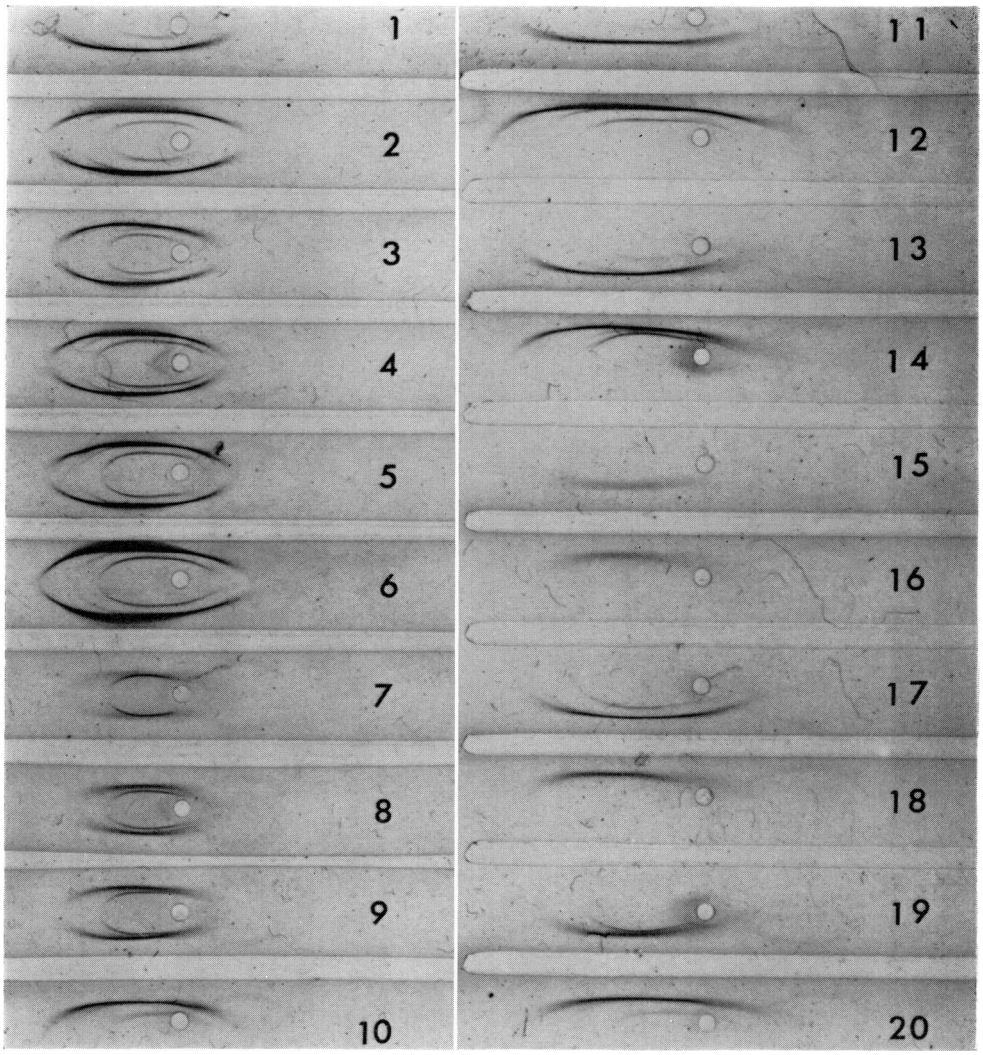

FIG. 9. Immunoelectrophoretic patterns given by sera from NZB donors and recipients of Series A (1–10) and B (11–20) with an antiserum against both IgM and IgG. (1) C3H/He normal adult pool; (2) NZB female donor A age 50 weeks; (3) NZB female recipient pass 1 age 104 days; (4) NZB female control age 99 days; (5) NZB male recipient pass 2 age 172 days; (6) NZB female control age 164 days; (7) NZB male recipient pass 3 age 32 days; (8) NZB male control age 29 days; (9) NZB male recipient pass 4 age 29 days; (10) C3H/He normal adult pool; (11) C3H/He normal adult pool; (12) NZB female donor B age 57 weeks; (13) NZB female recipient pass 1(a) age 157 days; (14) NZB female control age 174 days; (15) NZB male recipient pass 2(b) age 76 days; (16) NZB female recipient pass 2(a) age 60 days; (17) NZB male control age 69 days; (18) NZB male recipient pass 3(b) age 45 days; (19) NZB female control age 39 days; (20) C3H/He normal adult pool.

(sera 11 and 20). With the exception of the two original donors (sera 2 and 12) the sera of the majority of the NZB mice contained similar amounts of IgA irrespective of series or treatment but less than C3H/He mice. Neither did the IgG levels of the NZB recipients differ consistently from those of their controls. The only factor common to the three mice in Series A whose sera (sera 7–9) contained small amounts of IgG was that they were the

TABLE 3. Quantitative assessment of immunoglobulins present in the sera of intact NZB recipient mice using agar-gel diffusion

Serum No.	Sex	Age (days)	Treatment	Reciprocal titre†	
				IgM	IgA
Series A					
1*	Male and female	Adult	Untreated C3H/He	8	8
2	Female	354	Donor NZB	16	32
3	Female	104	Pass 1	8	4
4	Female	99	Control	16	8
5	Male	172	Pass 2	16	4
6	Female	164	Control	—	—
7	Male	32	Pass 3	32	1
8	Male	29	Control	16	1
9	Male	29	Pass 4	16	1
10*	Male and female	Adult	Untreated C3H/He	—	—
Series B					
11*	Male and female	Adult	Untreated C3H/He	8	8
12	Female	403	Donor NZB	32	32
13	Female	157	Pass 1(a)	8	4
14	Female	174	Control	32	4
15	Male	76	Pass 2(b)	4	2
16	Female	60	Pass (2a)	1	1
17	Male	69	Control	16	1
18	Male	45	Pass 3(b)	2	1
19	Female	39	Control	16	2
20*	Male and female	Adult	Untreated C3H/He	8	8

* Pooled sera used.
† See 'Materials and methods'.

youngest examined but this did not apply to the two passage animals in Series B (sera 15 and 16) which also showed low levels.

If the eight intact, untreated, NZB mice (including the donors) are regarded as a single group (sera 2, 4, 6, 8, 12, 14, 17 and 19), their sera contained more IgM but usually less IgA than C3H/He mice. Within the NZB group, despite an age span of 29–403 days, there was no marked change in IgM content attributable to sex, histological or clinical condition, so confirming our original observation (East *et al.*, 1965). IgG levels showed a slight tendency to increase as the animals aged and the greatest amount of IgA was found in sera of the two very old original donors.

DISCUSSION

Our finding that serial passage of spleen or lymph node tissue from four old, sick, untreated, Coombs positive, NZB donors causes an extensive reticulum cell neoplasia affecting the spleen, lymph nodes, lungs and liver of young syngeneic recipients can be interpreted in several ways. It could be that malignant reticulum cells, present among the more heterogeneous donor population, are placed at a proliferative advantage and disseminate quickly and extensively when transferred to young recipients. Alternatively, a transmissible agent, carried by the donor's cells, could provoke proliferation of the recipients' own cells. There is also a third possibility, namely that a transmissible agent latent in the recipients is activated by the treatment given.

It is interesting to note a brief comment by Holmes (1965) that four NZB mice injected at 4–5 weeks with spleen cells from 10- to 12-month-old NZB donors and killed 1 month later had enlarged spleens caused by proliferating reticulum cells. This author also stated that germinal centres were found in the thymic cortices of the four recipients. However, none of the forty-seven intact recipients examined in our experiments have shown such lesions, indeed the thymus has been relatively unaffected by treatment. Mellors (1966), too, reported malignant lymphomas in four NZB females aged 9–11 months. The lymphomas were classified either as reticulum cell sarcomas or pleomorphic in type and one sarcoma was transplanted successfully in NZB recipients.

In neither donors nor recipients of our successful Series A–D were the bone marrow, thymus or kidneys heavily infiltrated by reticulum cells and only a few appeared in the blood. We can offer no explanation as to why these organs, and the thymus-dependent areas in the lymph nodes of neonatally thymectomized recipients, were relatively exempt from the neoplastic process. Such exemption did not apparently apply to the eosinophils which proliferated in the recipients of Series E. The unsuccessful passage of bone marrow cells, spleen cells and ascites fluid from four other ageing donors could imply either that insufficient cells of the appropriate type were present in the inocula or that the tissues injected were not infective and that the age of the recipients may be an important parameter.

The spontaneous occurrence of reticulum cell neoplasms in old, Coombs positive, NZB mice, calls to mind the association of autoimmune haemolytic anaemia in patients with diseases of the lymphoreticular system which has lead to speculation that autoimmunity and certain forms of leukaemia are fundamentally either the same disease or different expressions of the same disturbance (Dameshek & Schwartz, 1959; Dacie, 1962; Dameshek, 1966). The fact that haemolytic episodes may follow virus pneumonia has also prompted the suggestion that virus infections may act as 'triggers' for human autoimmune disease (Dacie, 1962; van Loghem, 1965), while Stanley & Walters (1966), working with reovirus three in mice, have proposed that the virus transforms cells during the acute phase of the infection and that these cells later react against the host, leading either to an autoimmune disease or to malignancy.

The presence of numerous particles resembling murine leukaemia virus in the thymus and/or spleen or bone marrow of embryonic and young untreated NZB mice is, therefore, a challenging observation. Nevertheless, this finding must be regarded with some circumspection because spleen cell suspensions from young donors have not, to date, produced any malignant changes after inoculation into newborn, syngeneic recipients, while the spleen

cells used in the successful series of passages were obtained from old animals in which only a few particles have been seen in the thymus. Although particles were readily identified in the thymus and spleen and in serum and plasma pellets of the recipients of the successful passages, the mice did not develop prematurely positive antiglobulin reactions, suggesting that the autoimmune state was not, under the conditions of our experiments, a concomitant of successful transfer, and that the proliferating cells, whatever their origin, were not producing autoantibody. It was more difficult to assess the effect of passage on IgM content but the serum IgA and IgG levels of the recipients were not seriously distorted.

A direct link between the presence of the virus-like particles and the spontaneous neoplasia in NZB mice must await the outcome of experiments using cell-free filtrates. These experiments are now in progress but, as yet, filtrates prepared from the enlarged spleens of passage recipients have not affected newborn NZB mice. This may simply be a practical problem related to the titre of any infective virus obtained but it could also have important theoretical implications. Thus, we would interpret our results as indicating that the reticulum cell neoplasms of the recipient mice were produced by the passage of transformed cells from the old, untreated, donors. It is possible, but not proven, that virus, gradually increasing in titre, initiated the malignant transformation of the donors' cells but thereafter its presence was no longer necessary. We would hesitate, in view of the fact that the majority of our recipient mice remained Coombs negative, to assume that the particles present in untreated NZB mice are responsible for the autoimmune symptoms which characterize the strain, or that the autoimmune condition is directly related to the subsequent spontaneous malignancy.

The spontaneous occurrence of autoimmune reactions and transplantable reticulum cell neoplasms in NZB mice and the presence of particles resembling leukaemia virus in their tissues certainly provides a laboratory model for testing the attractive but theoretical proposition that autoimmunity, malignancy and virus infections are associated phenomena. However, direct relationships between any or all of these events have yet to be demonstrated experimentally.

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REFERENCES

BIELSCHOWSKY, M., HELYER, B.J. & HOWIE, J.B. (1959) Spontaneous haemolytic anaemia in mice of the NZB/BL strain. *Proc. Univ. Otago med. Sch.* **37,** 9.

BURNET, F.M. & HOLMES, M.C. (1964) Thymic changes in the mouse strain NZB in relation to the autoimmune state. *J. Path. Bact.* **88,** 229.

DACIE, J.V. (1962) *The Haemolytic Anaemias: Congenital and Acquired.* Part 2. *The Autoimmune Anaemias.* Churchill, London.

DAMESHEK, W. (1966) Immunocytes and immunoproliferative disorders. *The Thymus: Experimental and Clinical Studies.* Ciba Foundation Symposium, p. 399. Churchill, London.

DAMESHEK, W. & SCHWARTZ, R. (1959) Leukaemia and autoimmunization—Some possible relationships. *Blood*, **14**, 1131.

EAST, J. & PARROTT, D.M.V. (1962) Operative techniques for newborn mice using anaesthesia by cooling. *J. Endocrinol.* **24**, 249.

EAST, J. & DE SOUSA, M.A.B. (1966) The thymus, autoimmunity and malignancy in New Zealand Black mice. *Nat. Cancer Inst. Mongr.* **22**, 605.

EAST, J., PARROTT, D.M.V., CHESTERMAN, F.C. & POMERANCE, A. (1963) The appearance of a hepatotrophic virus in mice thymectomized at birth. *J. exp. Med.* **118**, 1069.

EAST, J., DE SOUSA, M.A.B. & PARROTT, D.M.V. (1965) The immunopathology of New Zealand Black (NZB) mice. *Transplantation*, **3**, 711.

FAHEY, J.L., WUNDERLICH, J. & MISHELL, R. (1964) The immunoglobulins of mice. *J. exp. Med.* **120**, 223.

GELL, P.G.H. (1957) The estimation of the individual human serum proteins by an immunological method. *J. clin. Path.* **10**, 67.

HELYER, B.J. & HOWIE, J.B. (1961) Positive lupus-erythematosus tests in a cross-bred strain of mice. *Proc. Univ. Otago med. Sch.* **39**, 17.

HELYER, B.J. & HOWIE, J.B. (1963) Spontaneous autoimmune disease in NZB/BL mice. *Brit. J. Haemat.* **9**, 119.

HOLMES, M.C. (1965) Coombs test conversion in young NZB mice induced by transfer of lymphoid cells from Coombs positive donors. *Aust. J. exp. Biol. med. Sci.* **42**, 589.

HOLMES, M.C. & BURNET, F.M. (1963) The natural history of autoimmune disease in NZB mice. *Ann. intern. Med.* **59**, 265.

LONG, G., HOLMES, M.C. & BURNET, F.M. (1963) Autoantibodies produced against mouse erythrocytes in NZB mice. *Aust. J. exp. Biol. med. Sci.* **41**, 315.

MELLORS, R.C. (1965) Autoimmune disease of NZB/BL mice. I. Pathology and pathogenesis of a model system of spontaneous glomerulonephritis. *J. exp. Med.* **112**, 25.

MELLORS, R.C. (1966) Autoimmune disease in NZB/BL mice. Autoimmunity and malignant lymphoma. *Blood*, **27**, 435.

MILLONIG, G. (1961) The advantage of a phosphate buffer for OsO_4 solutions in fixation. *J. appl. Physiol.* **32**, 1637.

PARROTT, D.M.V., DE SOUSA, M.A.B. & EAST, J. (1966) Thymus-dependent areas in the lymphoid organs of neonatally thymectomized mice. *J. exp. Med.* **123**, 191.

SCHEIDEGGER, J.J. (1955) Une micro-méthode de l'immuno-electrophorèse. *Int. Arch. Allergy*, **7**, 103.

STANLEY, N.F. & WALTERS, N-I.M. (1966) Virus induction of autoimmune disease and neoplasia. *Lancet*, **i**, 962.

VAN LOGHEM, J.J. (1965) Concepts on the origin of autoimmune diseases. The possible role of viral infection in the aetiology of idiopathic autoimmune diseases. *Series Haematol.* **9**, 1.

WIEME, R.J. (1959) *Studies on Agar-Gel Electrophoresis: Techniques—Applications.* Arsica Uitgaven N.V. Brussels.