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PARTICLES RESEMBLING MURINE LEUKAEMIA VIRUS IN NEW ZEALAND BLACK MICE

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SUMMARY

Particles which morphologically resembled murine leukaemia virus were detected by electron microscopy in the tissues (spleen, thymus, inguinal lymph nodes, bone marrow or pancreas) but not in serum or plasma pellets of untreated conventional New Zealand Black (NZB) mice aged 1–82 weeks. They were also found in the corresponding tissues of NZB mice which had been thymectomized shortly after birth. The presence of similar particles in the spleen, thymus or pancreas of conventional NZB embryos and, additionally, in the lymph nodes of NZB mice which had originally been introduced into a germ-free environment by Caesarian section and fostering on germ-free mice of another strain, suggests that the virus is transmitted 'vertically' through the germ cells or placenta. Preliminary investigations showed similar particles in the organs of conventional F_1 (NZB × NZW) hybrid and New Zealand White (NZW) mice.

Large numbers of particles also resembling murine leukaemia virus were found in the spleen and in plasma or serum pellets of young conventional NZB mice which had developed reticulum cell neoplasia following serial passage of lymphoid cell suspensions from ageing conventional NZB donors.

The possible relationship of these particles to the autoimmune reactions and malignant changes which occur spontaneously in conventional NZB mice is discussed.

INTRODUCTION

Conventional mice of the New Zealand Black (NZB) strain are immunologically aberrant and spontaneously develop a complex of pathological conditions. They produce large amounts of 19S macroglobulin throughout life (East, de Sousa & Parrott, 1965), they show positive antiglobulin (Coombs) reactions from the age of 4–5 months (Holmes & Burnet, 1963) and antinuclear factor occurs in their sera (Norins & Holmes, 1964). They also develop severe haemolytic anaemia (Bielschowsky, Helyer & Howie, 1959) and the ageing animals show gross splenomegaly and lymphadenopathy caused by extensive proliferations

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of plasma (Bielschowsky *et al.*, 1959; Helyer & Howie, 1963a) and/or reticulum cells (East *et al.*, 1965). Serial passage of lymphoid tissues from these ageing mice produces a lethal reticulum cell neoplasia in young NZB (East & de Sousa, 1966; East *et al.*, 1967a) or Balb/c (East & Prosser, 1967) recipients, while Mellors (1966) reported successful transplantation of a malignant lymphoma from donors aged 9–11 months. Renal disease, reminiscent of lupus nephritis, also occurs in NZB mice (Helyer & Howie, 1963b; Mellors, 1965; Hicks & Burnet, 1966) and is particularly severe in F_1 (NZB × NZW) hybrids (Helyer & Howie, 1963b; Hicks & Burnet, 1966).

The discovery of particles resembling murine leukaemia virus in the tissues of conventional (Mellors & Huang, 1966, 1967; Hollmann & Verley, 1966, 1967; Yumoto & Dmochowski, 1967; East *et al.*, 1967a) and germ-free (East *et al.*, 1967b) NZB mice and in NZB (East *et al.*, 1967a; Mellors & Huang, 1966) and Balb/c (East & Prosser, 1967) passage recipients has added yet another factor to the relationship between autoimmunity and malignancy. This paper presents more detailed and additional information on the presence of virus-like particles in untreated conventional and germ-free NZB mice, and in conventional NZB passage recipients. A preliminary account of their existence in conventional neonatally thymectomized NZB mice and conventional untreated NZW and F_1 (NZB × NZW) hybrid mice is also presented.

MATERIALS AND METHODS

Mice

Material for electron microscopic examination (usually spleen, inguinal lymph node, thymus, bone marrow, pancreas and plasma or serum) was obtained from untreated conventional NZB mice, including two 18-day embryos and fourteen mice aged 1–82 weeks inbred and maintained in this laboratory. One NZB mouse aged 16 weeks, thymectomized 36 hr after birth, and one NZB mouse aged 64 weeks, incompletely thymectomized 24 hr after birth, were also investigated.

Similar tissues were taken from three germ-free NZB mice aged 26, 34 and 45 weeks, bred at Carworth Incorporated, Rockland, U.S.A. and from one conventional F_1 (NZB × NZW) hybrid and one conventional NZW mouse supplied by the M.R.C. Rheumatism Research Unit, Taplow, Berks, and aged 12 and 4 weeks, respectively.

Spleen tissue and/or serum or plasma pellets were prepared from eleven moribund conventional recipient NZB mice aged 5–7 weeks which had developed reticulum cell neoplasia after serial passage of lymphoid cell suspensions originating from four old NZB donors designated A–D.

Control material from conventional mice of other strains was taken for comparison, including three C3H/Bi mice and two C57BL mice from this laboratory and two Balb/c mice supplied by the Department of Cancer Research, The London Hospital Medical College.

Methods

Approximately 1 ml of blood was obtained from each mouse. Plasma or serum pellets were prepared by ultracentrifugation as described previously (East *et al.*, 1967a). The pellets prepared from two passage recipient mice were negatively stained by resuspending with a few drops of 4% sodium silicotungstate and examined directly by electron microscopy.

Electron microscopy

Small blocks of tissue or pellet were fixed either in 1% OsO_4 buffered according to Millonig (1961) or Palade (1952) or in buffered 6·5 or 1·5% gluteraldehyde and post-fixed in 1% OsO_4, and embedded in methacrylate or epon. All blocks were stained in 0·5% uranyl nitrate before embedding in methacrylate. Epon sections were stained with a saturated aqueous solution of uranyl nitrate for 2 hr, followed by lead hydroxide for 2 min (Karnovsky, 1961). Section were cut on a Porter–Blum MT I or MT II ultramicrotome and examined in a Siemens Elmiskop I at 80 kV and at 10000 × magnification. Only a few grids of sections were taken from each block but a maximum of ten blocks of tissue or six blocks of pellet were examined.

RESULTS

Untreated conventional NZB mice

Particles, which morphologically resembled murine leukaemia virus, were found in the spleen, pancreas or thymus (Fig. 1) of 18-day embryos, and in the thymus, spleen, inguinal

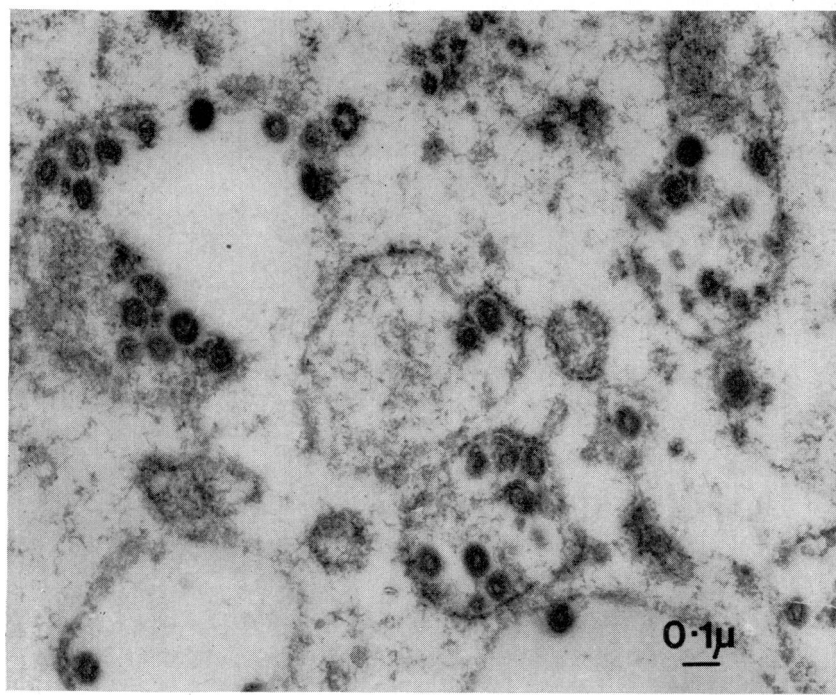


FIG. 1. Type C and type A1 particles present within cytoplasmic vacuoles of an 18-day embryo conventional NZB mouse, ×48,000. Fixed in 1% Millonig's OsO_4 and embedded in methacrylate.

lymph node and pancreas of most mice aged 1–82 weeks. These particles had an external unit membrane of average diameter 109 mμ (range 80–131 mμ) and a dense central nucleoid of average diameter 67 mμ. The frequency and number of particles occurring in the untreated conventional mice is shown in Table 1. It appears from this table that particles occurred more

frequently in the spleens of young, 10-week-old, healthy, Coombs negative, mice (Fig. 3) than in the spleens of older, sick, Coombs positive mice. This could be due to sampling error, because the spleens of the older mice were grossly enlarged, by extensive proliferation of

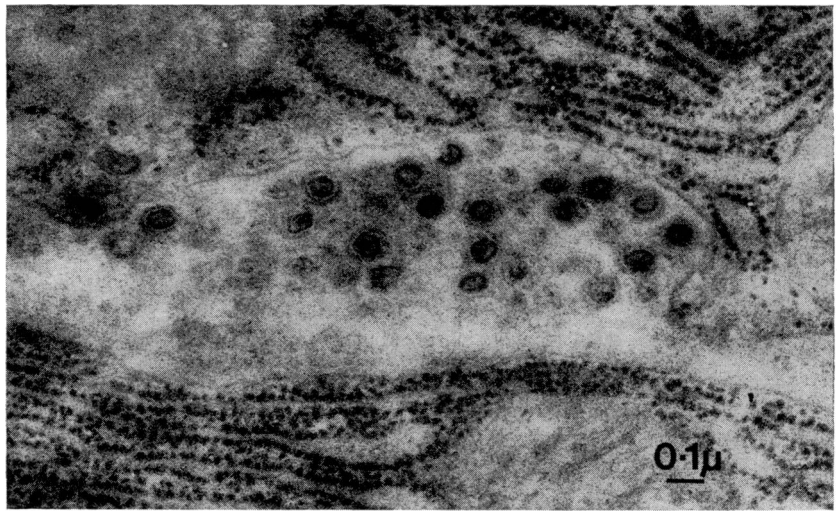


FIG. 2. Virus-like particles in the pancreas of a 45-week-old untreated germ-free NZB mouse. ×48,000. Fixed in 6·5% glutaraldehyde, post fixed in 1% Palade OsO_4 and embedded in epon.

TABLE 1. Particles resembling murine leukaemia virus in untreated conventional NZB mice: number of blocks containing particles/number of blocks examined

Mouse	Spleen	Thymus	Inguinal lymph node	Bone marrow	Pancreas	Serum or plasma	Coombs test
18-day embryo	0/6	1/3(3)	—	—	—	—	—
18-day embryo	2/3(2)	—	0/1	—	1/4(2)	—	—
Male, 1 week	1/7(1)	1/3(2)	—	—	—	0/5	—
Male, 10 weeks	2/4(3, 3)	1/9(3)	2/3(3, 2)	2/5(1, 1)	—	0/5	Negative
Male, 10 weeks	3/5(2, 3, 3)	—	—	—	—	—	Negative
Female, 26 weeks	0/8	1/3(2)	1/3(3)	2/3(2)	—	—	Positive
Male, 56 weeks	1/8(1)	1/5(2)	—	1/6(1)	—	0/5	Positive
Female, 64 weeks	0/6	1/5(3)	1/7(3)	—	2/2(3, 3)	—	Positive
Male, 78 weeks	1/8(1)	—	—	1/6(1)	—	0/4	Positive
Male, 82 weeks	2/8(2, 2)	2/6(2, 2)	1/5(1)	2/5(1, 2)	—	—	Positive

(1) One particle; (2) several particles; (3) very many particles.

plasma and/or reticulum cells (East *et al.*, 1967a). No particles were detected in plasma or serum pellets prepared from four of these mice (Table 1), nor in pellets prepared from six other untreated conventional NZB mice aged 6–70 weeks.

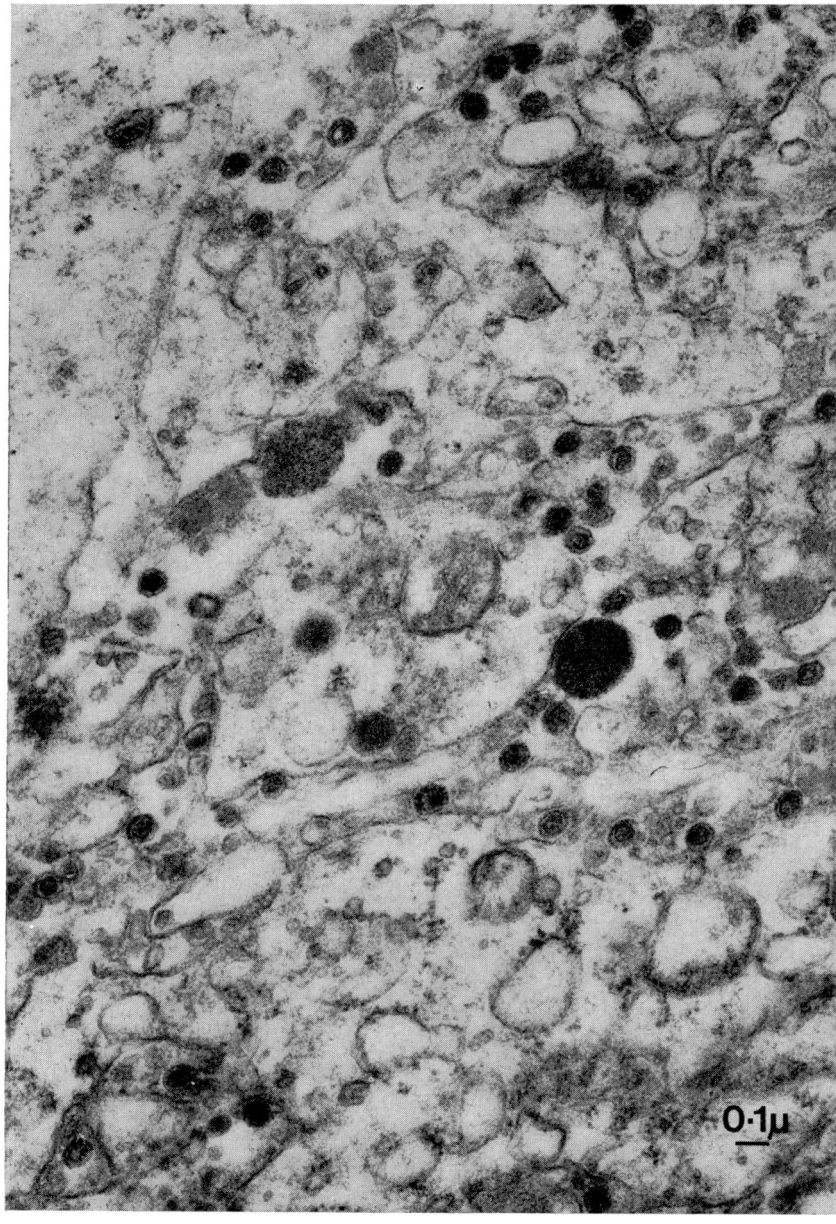


FIG. 3. Virus-like particles amongst cytoplasmic processes in the spleen of a 10-week-old untreated conventional NZB mouse, ×40,000. Fixed in 1% Millonig's OsO_4 and embedded in methacrylate.

Thymectomized conventional NZB mice

Virus-like particles were again detected in the spleen, inguinal lymph node, bone marrow or pancreas of two mice aged 16 weeks and 64 weeks (Table 2). The latter animal, partially thymectomized 24 hr after birth, was unusual in that particles were easily detected in the thymic remnant and in all the organs examined, and a few particles were present in the plasma pellet.

TABLE 2. Particles resembling murine leukaemia virus in thymectomized conventional NZB mice: number of blocks containing particles/number of blocks examined

Mouse	Spleen	Thymus	Inguinal lymph node	Bone marrow	Pancreas	Serum or plasma	Coombs test
Male, 16 weeks, thymectomized 36 hr after birth	0/8	—	3/6[2, 2, 3]	0/6	3/3[3, 3, 3]	—	Negative
Female, 64 weeks, partially thymectomized 24 hr after birth	4/6[1, 1, 2, 2]	2/4[2, 2] Thymic remnant	1/3[3]	1/3[2]	—	1/3[2]	Positive

[1] One particle; [2] several particles; [3] very many particles.

Untreated germ-free NZB mice

Similar particles were observed in the lymph node of the 26-week-old Coombs negative mouse and in the spleen, thymus, inguinal lymph node or pancreas (Fig. 2) of the other two Coombs positive mice (Table 3). The average external diameter of the particles was 103 mμ; the average diameter of the nucleoid was 67 mμ.

TABLE 3. Particles resembling murine leukaemia virus in untreated germ-free NZB mice: number of blocks containing particles/number of blocks examined

Mouse	Spleen	Thymus	Inguinal lymph node	Bone marrow	Pancreas	Serum or plasma	Coombs test
Male, 26 weeks	0/6	—	1/3[3]	—	—	—	Negative
Female, 34 weeks	1/6[1]	0/6	2/3[3, 3]	—	—	—	Positive
Male, 45 weeks	0/6	1/5[3]	1/6[3]	—	1/1[3]	—	Positive

[1] One particle; [2] several particles; [3] very many particles.

Untreated conventional NZW and F_1 (NZB × NZW) mice

Preliminary examination has also revealed the presence of particles resembling murine leukaemia virus in the spleen and pancreas of a hybrid mouse, and in the spleen, pancreas and thymus (Fig. 4) of the NZW mouse (Table 4).

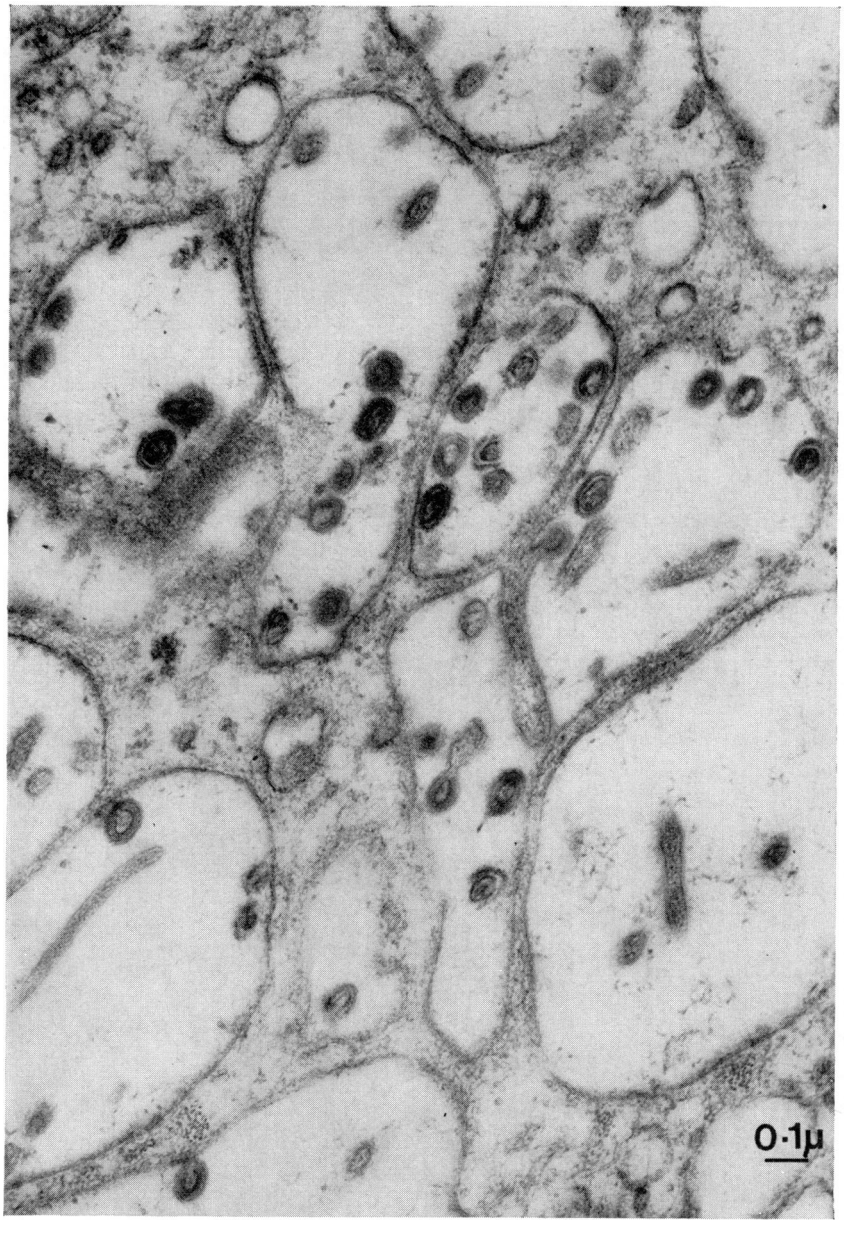


FIG. 4. Type C and type A1 particles within cytoplasmic vacuoles in the thymus of an untreated conventional NZW mouse aged 4 weeks, ×56,000. Fixed in 1% Palade OsO_4 and embedded in methacrylate.

TABLE 4. Particles resembling murine leukaemia virus in untreated conventional NZW and F_1 (NZB × NZW) mice: number of blocks containing particles/number of blocks examined

Mouse	Spleen	Thymus	Inguinal lymph node	Bone marrow	Pancreas	Serum or plasma
F_1 (NZB × NZW) female, 12 weeks	2/6[1, 3]	—	—	—	1/1[2]	—
NZW female, 4 weeks	1/4[2]	2/3[1, 3]	0/3	0/3	2/4[2, 1]	—

[1] One particle; [2] several particles; [3] very many particles.

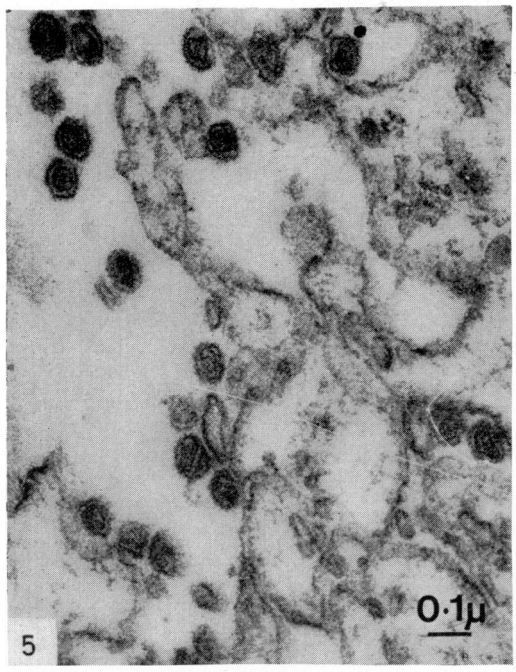
Recipient conventional NZB mice

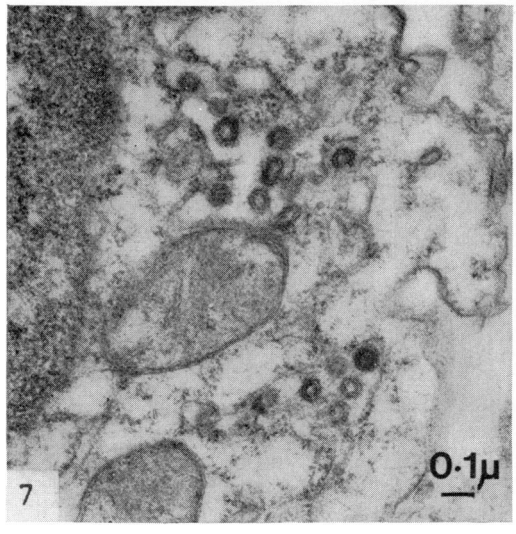
Eleven young conventional NZB mice, which had developed a lethal and generalized reticulum cell neoplasia after serial passage of spleen or lymph node suspensions from four ageing conventional NZB donors, were examined (Table 5). All these recipients remained Coombs negative.

TABLE 5. Particles resembling murine leukaemia virus in recipient conventional NZB mice: number of blocks containing particles/number of blocks examined

Recipient	Spleen	Thymus	Inguinal lymph node	Bone marrow	Pancreas	Serum or plasma	Coombs test
Male, 6 weeks Pass 3 donor B	2/2[2, 3]	—	—	—	—	—	Negative
Male, 6 weeks Pass 3 donor B	1/1[3]	—	—	—	—	—	Negative
Male, 6 weeks Pass 3 donor B	3/3[3, 1, 2]	—	—	—	—	—	Negative
Male, 7 weeks Pass 3 donor B	—	—	—	—	—	5/5[3, 3, 3, 3, 3]	Negative
Female, 7 weeks* Pass 3 donor D	1/7[2]	2/5[2, 2]	5/5[1, 2, 3, 2, 1]	2/3[2, 2]	—	—	Negative
Female, 7 weeks Pass 3 donor D	—	—	—	—	—	1/4[2]	Negative
Male, 4 weeks Pass 4 donor C	1/5[1]	—	—	—	—	3/4[2, 2, 2]	Negative
Female, 7 weeks Pass 4 donor B	3/3[3, 3, 3]	—	—	—	—	Negative staining[3]	Negative
Female, 7 weeks Pass 4 donor B	1/1[3]	—	—	—	—	Negative staining[3]	Negative
Male, 5 weeks Pass 5 donor C	—	—	—	—	—	1/2[2]	Negative
Female, 5 weeks Pass 10 donor A	3/3[2, 2, 2]	2/2[2, 2]	3/3[3, 3, 3]	0/2	—	3/3[3, 3, 3]	Negative

[1] One particle; [2] several particles; [3] very many particles.
*Mainly 'doughnut' type particles.





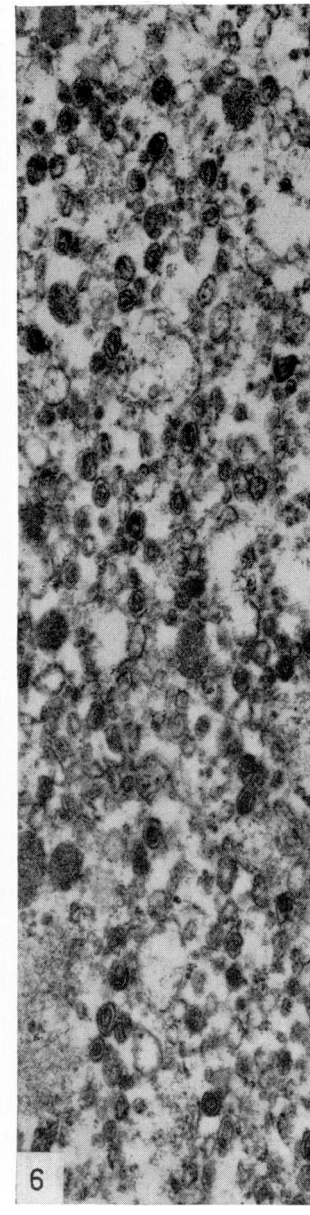


FIG. 5. Particles resembling murine leukaemia virus in the spleen of a 7-week-old conventional recipient NZB mouse, which had been inoculated with fourth passage lymphoid cells, × 56,000. Fixed in 1% Millonig's OsO_4 and embedded in methacrylate.

FIG. 6. Numerous virus-like particles in a thin section of a plasma pellet prepared from a 5-week-old conventional recipient NZB mouse which had been inoculated with tenth passage lymphoid cells, × 32,000. Fixed in 1% Millonig's OsO_4 and embedded in methacrylate.

FIG. 7. Intracytoplasmic 'doughnut type' particles from the spleen of a 7-week-old conventional recipient NZB mouse which had been inoculated with third passage lymphoid cells, × 40,000. Fixed in 1% Millonig's OsO_4 and embedded in methacrylate.

Particles resembling murine leukaemia virus occurred in great numbers in the enlarged spleens of most of the recipient mice. They were present in intercellular spaces (Fig. 5) and often budding from the surface of abnormal cells. Another striking observation was the presence of numerous virus-like particles in the plasma or serum pellets of the recipients. The negatively stained plasma pellets showed similar 'tailed' particles as described by Dalton, Haguenau & Moloney (1962) for unfixed preparations of Moloney virus (Fig. 8).

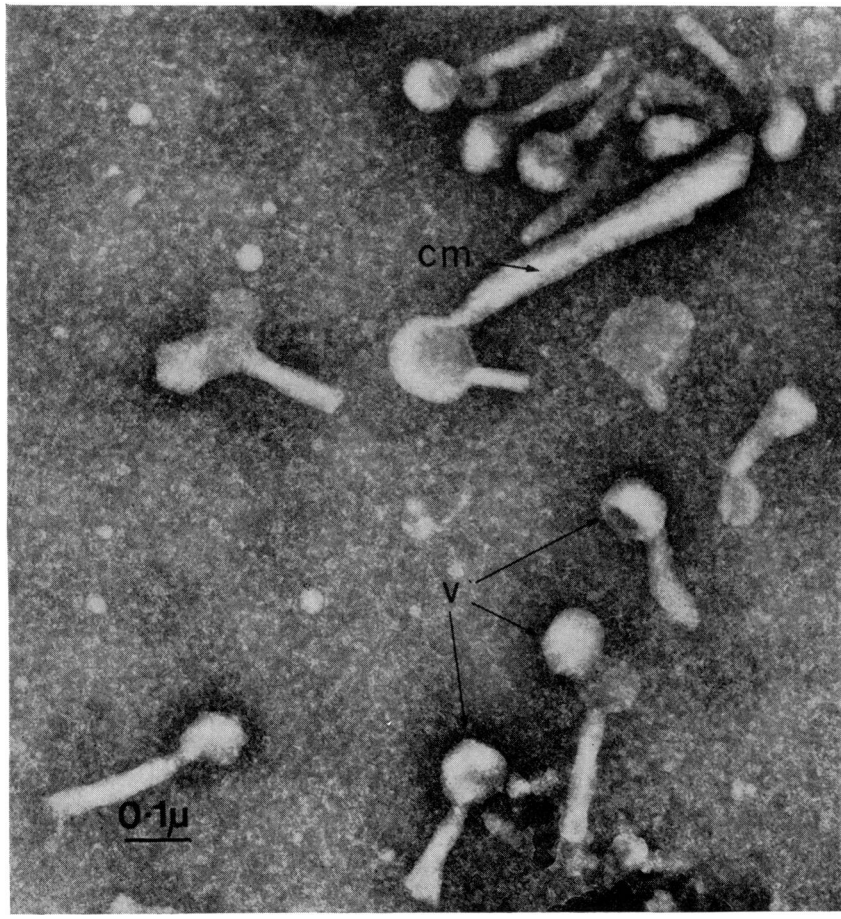


FIG. 8. Plasma pellet, negatively stained with 4% sodium silicotungstate, prepared from an 8-week-old conventional recipient NZB mouse which had been inoculated with fourth passage lymphoid cells, × 80,000. (V, Virus-like particles; CM, cell membrane fragment.)

Most particles were of type C form (Bernhard, 1960), though occasionally intracellular 'doughnut' type particles with double membranes, but no dense nucleoid, occurred in the tissues. In one mouse this was the main type seen (Fig. 7). The average external diameter of the type C particles was 103 mμ (range 80–129 mμ).

The distribution of particles in the spleens of the recipients differed from that observed in young untreated NZB mice of a comparable age, i.e. 10 weeks. In the latter, many virus-like particles were also seen but these were concentrated around cytoplasmic processes of

'reticular' cells. On the other hand, very few particles were detected in the spleens of older untreated NZB mice of equivalent age to the original donors of the passage material. Neither young nor old untreated NZB mice had particles in their serum or plasma yet pellets prepared from the recipient mice contained very large numbers of particles indeed.

Untreated conventional C3H/Bi, C57BL and Balb/c mice

A similar search through the tissues of one C3H/Bi and two Balb/c mice revealed only one intracellular virus-like particle in the inguinal lymph node of a Balb/c mouse. Particles were not detected in any other tissues nor in the serum or plasma pellets of two C57BL mice and three C3H/Bi mice (Table 6). However, it must always be borne in mind that virus particles can only be detected by electron microscopy when large numbers are present.

TABLE 6. Particles resembling murine leukaemia virus in untreated conventional C3H/Bi, C57BL and Balb/c mice: number of blocks containing particles/number of blocks examined

Mouse	Spleen	Thymus	Inguinal lymph node	Bone marrow	Pancreas	Serum or plasma
C57BL male, 7 weeks	—	—	—	—	—	0/5
C57BL female, 37 weeks	—	—	—	—	—	0/5
C3H/Bi female, 8 weeks	—	—	—	—	—	0/3
C3H/Bi female, 20 weeks	0/6	0/6	0/6	0/6	—	0/2
C3H/Bi male, 56 weeks	—	—	—	—	—	0/4
Balb/c male, 6 weeks	0/7	0/6	1/5[1]	0/3	0/5	—
Balb/c female, 6 weeks	0/6	0/6	0/4	0/5	0/5	—

[1] One particle.

Distribution of virus-like particles in the tissues of untreated conventional, germ-free and thymectomized NZB mice

The particles most frequently observed were of the type C form, although both type C and type A1 (de Harven, 1962) were found in the thymus. In this organ they were within cytoplasmic vacuoles of epithelial cells, and budding particles could be seen, (Fig. 1), though some extracellular particles were detected. Particles usually occurred in great numbers amongst cytoplasmic processes, of what were probably reticular cells, in the spleen of younger mice (Fig. 3) and in the lymph nodes of mice of all ages. The pancreas contained small clusters of particles around the edge of acinar cells (Fig. 2). Very few particles were observed in bone marrow; these were always extracellular and were never seen in megakaryocytes of untreated mice. The type B particles, similar to the mammary tumour agent, which Hollmann & Verley (1966, 1967) reported to occur in the thymus of their healthy NZB mice, have not been detected in any of our animals.

DISCUSSION

This report, though not intended to be a quantitative study, shows quite clearly that particles, morphologically similar to murine leukaemia virus, are present in organs of conventional NZB mice prior to birth and throughout life. Their presence in conventional NZB embryos and in germ-free NZB mice, which originated by Caesarian delivery and were fostered on germ-free mice of another strain, suggests that the virus-like particles are transmitted vertically via the placenta or germ cells. There have been several recent reports, from institutes other than our own, of virus-like particles occurring in conventional NZB mice (Mellors & Huang, 1966, 1967; Hollmann & Verley, 1966, 1967; Yumoto & Dmochowski, 1967) which would eliminate the possibility of a contaminant virus peculiar to this laboratory. It would be interesting to know the origin of this virus. The conventional NZB mice originated from a mixed population obtained from the Imperial Cancer Research Fund in 1930 and were selected initially only for coat colour by Dr Bielschowsky in New Zealand in 1948 (Bielschowsky & Bielschowsky, 1964). As we have found similar particles in NZW and F_1 (NZB × NZW) mice it seems possible that virus was present in the original parent stock. Virus-like particles have been found in untreated mice of other strains (de Harven, 1964; Masakiro & Pollard, 1965; Feldman & Gross, 1966) though these occurred mainly in the thymus, but Chapman *et al.* (1966) reported virus-like particles in lymphatic tissues of conventional CFW_w mice.

Virus-like particles were found in both Coombs negative and Coombs positive untreated NZB mice and, indeed, must have been present in their lymphoid tissues long before any autoimmune or neoplastic changes appeared. However, it is not yet possible to relate the presence of such particles to the autoimmune condition.

The only claim that cell free filtrates of NZB mouse tissues can transfer Coombs positivity is that of Mellors & Huang (1967) who reported positive indirect, but not direct, Coombs tests in five of twenty-nine Swiss mice inoculated. It should, however, be noted that these same authors could not evoke direct Coombs positive reactions in young NZB recipients with filtrates of a spontaneous malignant lymphoma (Mellors & Huang, 1966) or with spleen cell suspensions (Mellors, 1966).

The successful transfer of reticulum cell neoplasia from old conventional NZB donors to young NZB or Balb/c recipients by inoculation of spleen or lymph node suspensions has been reported previously and the presence of virus-like particles in both donors and recipients described (East & de Sousa, 1966; East *et al.*, 1967a; East & Prosser, 1967). It has now been shown that the particles occur in much greater numbers in the spleens of the NZB recipients than in those of old untreated NZB mice of equivalent age to the original donors. Moreover, the plasma or serum pellets of the NZB recipients contain vast numbers of particles, while those of untreated animals do not. However, none of the recipient mice became prematurely Coombs positive and cell free filtrates prepared from spleens and plasma of recipient mice have not yet induced a reticulum cell neoplasia (East, personal communication). Thus, to date, experimental data tends to favour a cell mediated, rather than viral induced neoplasia, though it is possible that virus, gradually increasing in titre, is responsible for initiating malignant transformation in the older NZB mice. It is interesting to note that Mellors & Huang (1966) using cell free filtrates prepared from a malignant lymphoma originating from an old NZB mouse, induced only pathological kidney changes in five pre-weanling recipients of the same strain.

Evidence of an immune response, indicated by the increased development of lymphoid and plasma cells and high immunoglobulin levels, is still apparent in NZB mice reared under germ-free conditions (East *et al.*, 1967b). One could suggest that the virus is the antigenic stimulus and that such an immunologically aberrant strain of mice would not be tolerant to a vertically transmitted virus.

Gross antigen has been identified in conventional NZB mouse tissues (Old & Boyse, personal communication) but in our opinion the neoplasia which develops spontaneously in our animals is not a typical lymphocytic Gross leukaemia. As the virus-like particles present in the NZB mice have not yet been proved to have oncognic activity, it is not possible to assess the significance of this finding. It seems unlikely that the particles which occur so frequently in the tissues of NZB mice and which morphologically resemble other murine leukaemia viruses, exist purely as passengers, but this possibility must be considered.

ACKNOWLEDGMENTS

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