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AUTOIMMUNE REACTIONS AND MALIGNANT CHANGES IN GERM-FREE NEW ZEALAND BLACK MICE

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SUMMARY

Although NZB mice were bred and maintained in a germ-free environment their spleens enlarged and showed a sequence of histological events concomitant with the advent of positive antiglobulin (Coombs) reactions at 8–10 months which were similar to, but less intense than, those of their conventional NZB counterparts. The numerous large follicles with prominent germinal centres which developed in the white pulp and the proliferations of large pyroninophilic cells in the red pulp thus represented a humoral autoimmune reaction uncomplicated by external microbial antigenic stimuli. This burst of immunological activity in the spleen was followed by a reticulum cell neoplasia (apparently originating within the follicles and from the perifollicular mantles) which was transferable by intraperitoneal injection of spleen cell suspensions to syngeneic and allogeneic (BALB/c) recipients. By comparison, the inguinal lymph nodes of these same germ-free NZB mice were both immunologically inactive and exempt from the malignant process. Lesions in the thymus, and kidney lesions resembling human membranous glomerulonephritis or lupus nephritis, were found in both germ-free and conventional mice of this strain. Possible relationships between the autoimmunity, malignancy and the virus-like particles known to be present in germ-free NZB mice are discussed.

INTRODUCTION

New Zealand Black (NZB) mice bred and maintained in a germ-free environment show the same stigma of spontaneous autoimmunity as their conventional counterparts, namely, positive antiglobulin (Coombs) reactions (East *et al.*, 1967a). Other immunological aberrations—the presence of serum antinuclear factor and a high level of circulating macroglobulin (IgM)—are also common to both (East *et al.*, 1967a). Moreover, the electron

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microscopic identification of type 'C' particles resembling murine leukaemia virus in germ-free (East *et al.*, 1967a; Prosser, 1968) as well as in conventional embryo and adult NZB mice (Mellors & Huang, 1966; East *et al.*, 1967c; Hollmann & Verley, 1967; Yumoto & Dmochowski, 1967; Prosser, 1968) has prompted speculation about their relationship to the autoimmunity and/or to the spontaneous transferable reticulum cell neoplasia which is expressed as gross splenomegaly and lymphadenopathy in ageing conventional animals of this strain (East & de Sousa, 1966; Mellors, 1966; East *et al.*, 1967c).

To our knowledge, no descriptive pathology of NZB mice isolated in a germ-free environment has yet been published. The unique advantage of such a study is that it permits histological definition of an autoimmune reaction uncomplicated by external antigenic stimulation. At the same time we were able to determine whether the kidney lesions, resembling human lupus nephritis, that occur in ageing conventional NZB mice (Helyer & Howie, 1963a; Hicks & Burnet, 1966) were to be found in the germ-free animals. The germ-free NZB mice were also examined for malignant changes both histologically and by passage of their lymphoid cells in conventional syngeneic or allogeneic recipients. Although the immunopathology of the conventional NZB mice maintained in these laboratories has already been reported in detail (East, de Sousa & Parrott, 1965; East *et al.*, 1967c) additional conventional animals were included for strict comparison and data were obtained from a small number of germ-free CFW mice as well as from conventional C3H/Bi mice.

MATERIALS AND METHODS

Animals

Conventional NZB mice originally imported at the fifty-third generation by the Laboratory Animals Centre, Carshalton, England, direct from Dr F. Bielschowsky, University of Otago, New Zealand (Bielschowsky, Heyler & Howie, 1959) formed the nucleus of the inbred conventional NZB colony now maintained at the Imperial Cancer Research Fund for a further sixteen generations, and also provided the basic stock for the germ-free colony. A total of twenty (eight female and twelve male) inbred germ-free NZB mice was investigated. These included twelve of the animals, aged 6–11 months, whose subsequent derivation and maintenance at Carworth Incorporated, Rockland, U.S.A. have already been described (East *et al.*, 1967a) and eight mice, aged 9–17 months, selected from the same germ-free colony 15 months later. Two germ-free 8-month-old Carworth Farm White (CFW) mice were also used. Comparative data were provided by fifty-five (twenty-six female and nineteen male) inbred conventional NZB mice and thirty-nine (eighteen female and twenty-one male) inbred conventional C3H/Bi mice aged 6–19 months, from the Imperial Cancer Research Fund colonies. Inbred BALB/c mice, supplied by the Cancer Research Department, London Hospital Medical College, acted as recipients in passage experiments.

Direct antiglobulin (Coombs) test

Mice were bled from the tail or retro-orbital plexus into heparin and the tests performed by the method already described (East *et al.*, 1965) using rabbit anti-mouse globulin (Microbiological Associates, U.S.A.) diluted 1:5, 1:25 and 1:125.

Packed cell volume (PCV)

Packed cell volumes were measured by the micro-haematocrit technique using blood from the tail or axilla.

Histological material

Material was available from fifteen (seven female and eight male) germ-free and sixteen (eleven female and five male) conventional NZB mice and from two germ-free CFW mice. Sections of spleen, thymus, inguinal and mesenteric lymph nodes, kidneys, lungs, liver and gut, were fixed in formol–saline, formol–acetic-alcohol or formol–ammonium bromide, and cut at 3–6 μ. They were stained routinely with haematoxylin and eosin and, when required, with methyl-green–pyronin, periodic acid–Schiff (PAS), van Gieson, Unna's orcein or picro-acid–Mallory (Lendrum *et al.*, 1962). Reticulin and reticulum cells were specifically stained using the methods of Gordon & Sweet and del Rio Hortega, respectively. Imprints were stained with May–Grüenwald–Giemsa.

Cell suspensions

Spleens were removed from germ-free NZB donors under sterile conditions, minced coarsely in sterile isotonic saline or phosphate-buffered saline (pH 7·2), and the suspensions passaged by intraperitoneal injection using a volume of 0·05–0·2 ml containing approximately $60–100 \times 10^6$ cells. One-day-old conventional NZB and BALB/c recipients were used for the first passages and older recipients thereafter.

RESULTS

Spleen weight (Fig. 1)

Spontaneous, often massive, splenomegaly was a characteristic of conventional NZB mice. Their spleens were just palpable at 6–8 months but, thereafter, enlarged to such an extent that, on average, they finally weighed 1·26 g (0·4–2·3 g). Splenomegaly also developed in the germ-free NZB mice, although rather later (at 8–10 months) and not so dramatically, but their average final spleen weight of 0·51 g (0·1–0·7 g) was still much greater than that of normal conventional C3H/Bi mice (0·15 : 0·09–0·26 g).

Packed cell volume (Table 1)

The packed cell volumes (PCV) of conventional and germ-free NZB mice were very similar at 6–8 months and did not alter until 9–10 months when some animals in both groups began to show signs of anaemia. There was always considerable variation but, ultimately, the PCVs of the conventional animals were more severely depressed and could fall as low as 17%. The haematocrits of the normal conventional C3H/Bi mice changed only slightly as they aged over a comparable period of time.

Antiglobulin (Coombs) tests

The antiglobulin tests of seven germ-free NZB mice were unequivocally positive at 10–17 months and one female was still negative at 9 months. This amply confirms our original report on twelve germ-free animals in which Coombs conversion occurred between 6 and 8

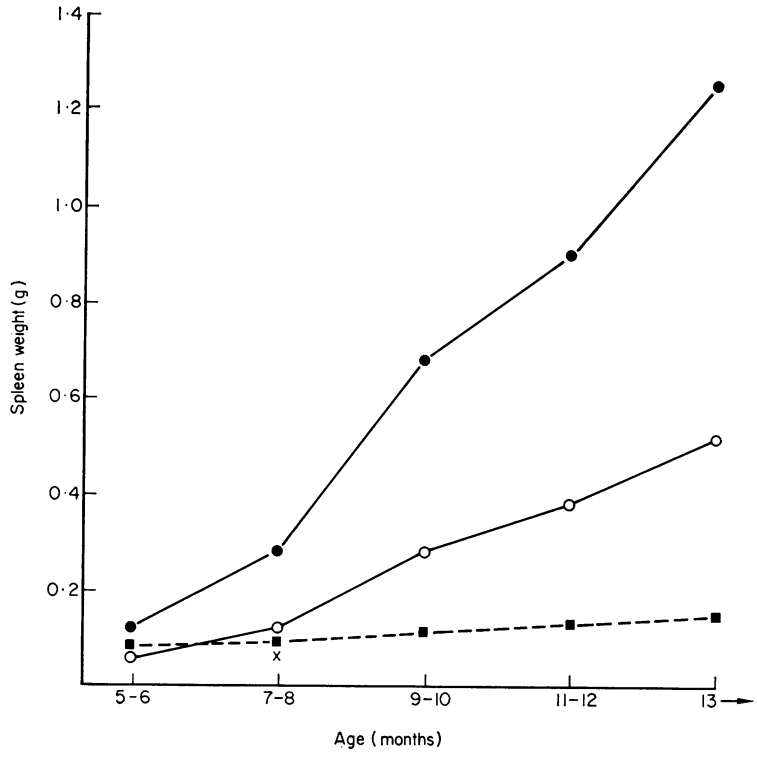

FIG. 1. Mean comparative spleen weights of fifty-five conventional NZB (●), twenty germ-free NZB (○), thirty-nine conventional C3H/Bi (■) and two germ-free CFW (×) mice.

TABLE 1. Packed cell volumes (PCV) of conventional and germ-free mice

Age (months)	Conventional C3H/Bi mice		Conventional NZB mice		Germ-free NZB mice	
	No. of mice	PCV% (mean and range)	No. of mice	PCV% (mean and range)	No. of mice	PCV% (mean and range)
6	10	45 (43–47)	10	44 (40–45)	4	44 (41–48)
8	10	43 (42–46)	10	43 (39–44)	4	44 (40–46)
9–10	8	42 (40–46)	9	35 (27–40)	4	34 (28–40)
11–17	11	41 (39–42)	16	29 (17–38)	8	38 (32–40)

months, slightly later than in conventional NZB mice. All the conventional C3H/Bi mice were Coombs negative.

Histology

Spleen. The spleens of the youngest (6-month) germ-free NZB mice were smaller than those of their conventional counterparts because both the white and the red pulp were underdeveloped. Nevertheless, follicles with germinal centres composed of large pyroninophilic cells, plasmablasts, plasma cells, cells in mitoses and cellular debris, were present in the white pulp while large pyroninophilic cells and a few mature plasma cells were also scattered

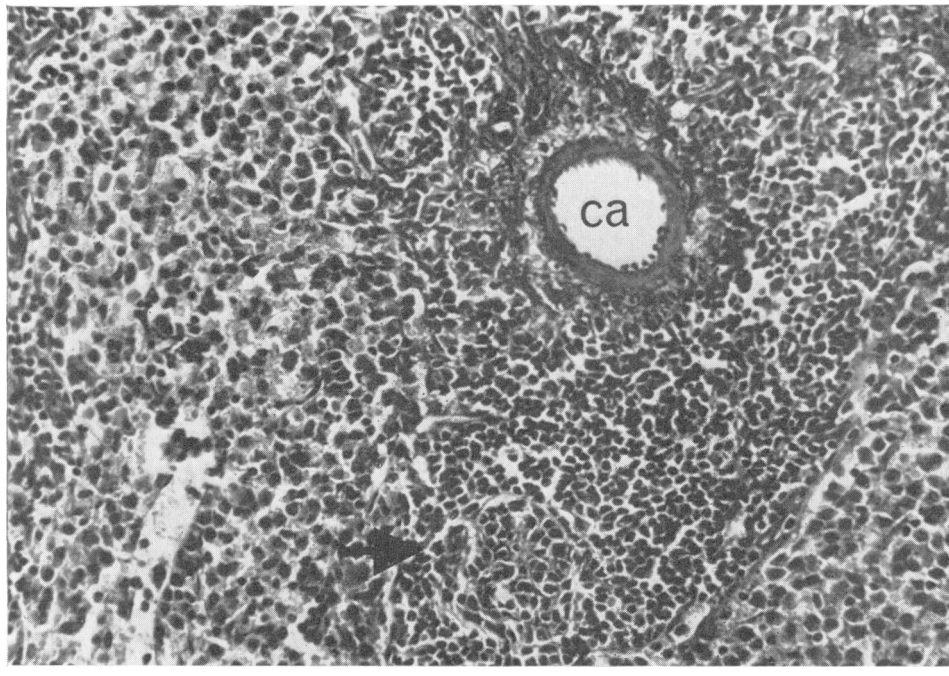

FIG. 2. Spleen of a germ-free NZB mouse aged 6 months. Note small germinal centre (→) thickening of dilated central arteriole (ca) and large (pyroninophilic) cells in red pulp. Lendrum, × 330.

diffusely throughout the red pulp (Fig. 2). However, the follicles were fewer, much smaller and less active than in the conventional NZB mice and there was little sign of the extensive haemopoiesis and megakaryocytosis which developed in such animals when 6–8 months old.

The spleens of the germ-free NZB mice enlarged concomitant with the onset of Coombs positivity at 8–10 months and as activity increased in the white and the red pulp. The follicles were now numerous and large with prominent germinal centres, but were rather indistinct in outline due to proliferations of reticulum cells in their perifollicular mantles. Simultaneously, huge numbers of large pyroninophilic cells appeared in the red pulp often localized around the arteries, along the trabeculae, and in the subcapsular zone, but mature plasma cells were still relatively scarce. Eosinophils were sometimes very prolific and haemopoiesis was well established. Megakaryocytes were most conspicuous and many big

epithelioid cells seemed to be in the process of fusing to form giant cells that resembled Reed–Sternberg cells (Fig. 3a). By comparison, the spleens of the 8-month-old germ-free CFW mice were extremely small, ill-differentiated, without germinal centres, but with a smattering of plasma cells in the red pulp.

The pattern of development changed in the germ-free NZB mice at 10 months as reticulum cells became more dominant appearing to disseminate from the perifollicular mantles into the red pulp (Fig. 4). Areas of haemopoiesis persisted. If we couple these findings with evidence obtained by the use of specific stains on conventional materials, we are tempted to make a dynamic interpretation from static observations. We suggest that immature cells

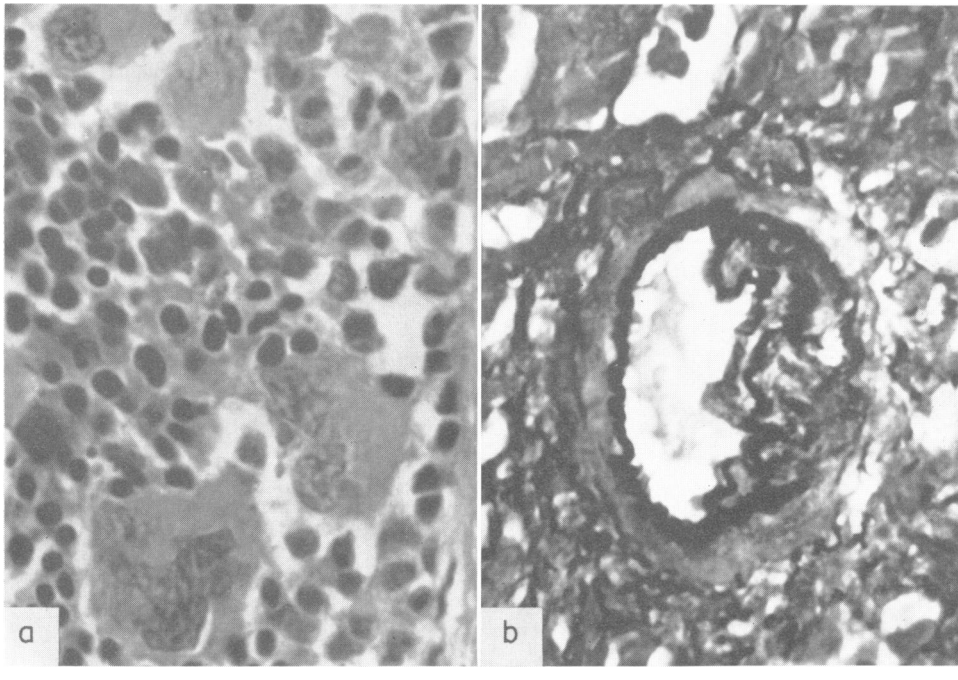

FIG. 3. (a) Splenic red pulp of a germ-free NZB donor aged 10 months. Many epithelial cells fusing to form cells of the Reed–Sternberg type. H & E, × 825. (b) Central follicular arteriole in the spleen of a germ-free NZB mouse aged 10 months showing splitting of internal and external laminas. Unna's orcein, × 840.

within the follicles proliferate, break through and mingle with the reticulum cells of the perifollicular mantles, and then disseminate into the red pulp where they initially pre-empt those positions previously occupied by the large pyroninophilic cells. In architecture and size the spleens of the older germ-free NZB mice could resemble those of the conventional animals but, in the latter, splenomegaly was usually more marked and the histological picture more exaggerated with maximal haemopoiesis and/or sheets of reticulum cells obliterating all structure.

The spleens of the germ-free NZB mice also exhibited marked vascular lesions involving the central arterioles of the follicles (Figs. 2 and 3b) and the septal arteries of the red pulp, although they were never as severe as in the conventional NZB animals. In 6-month-old

germ-free mice the arterial walls were thickened and the adventitial lamina infiltrated by lymphocytes. Subsequently, PAS-positive material gradually accumulated under the endothelial lining to split the internal elastic lamina and eventually involve the medial and external layers. Sclerosis and hyalinization of the vessels caused restriction or obliteration of their lumina (Fig. 3b) and, in some cases, the presence of granulomatous tissue around the degenerate arteries resembled fibrinoid necrosis.

Inguinal lymph nodes. Most unexpectedly the inguinal nodes of the germ-free NZB and CFW mice were very similar (Fig. 5b and c), and differed radically from those of NZB mice reared under conventional conditions (Fig. 5a). In the youngest germ-free NZB mice they were very small and seriously depleted of lymphocytes but did show sparse primary and

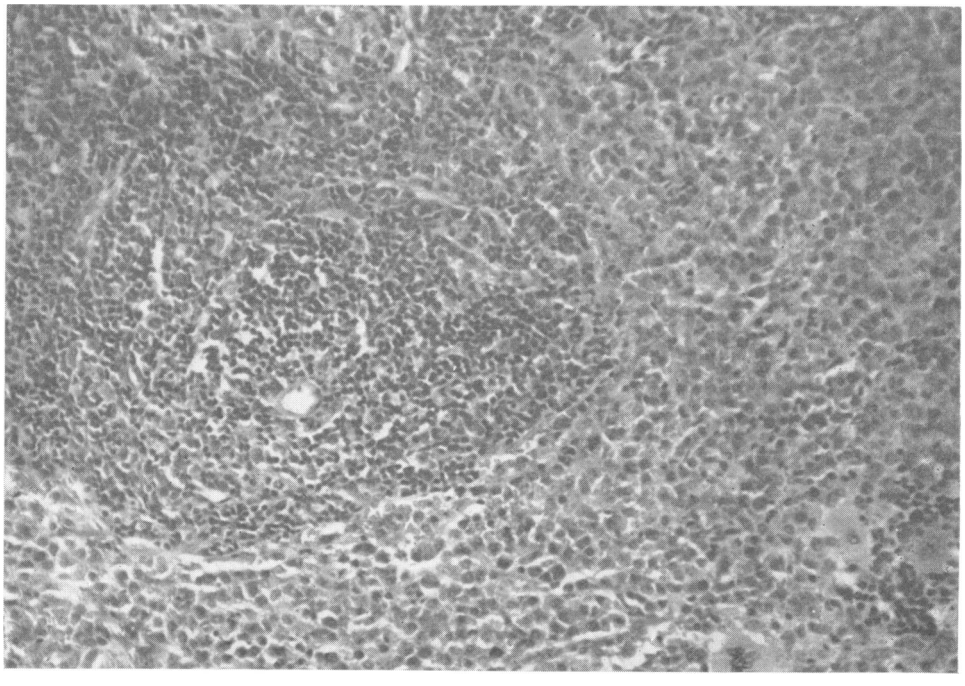

FIG. 4. Spleen of a germ-free NZB mouse aged 8 months showing dissemination of reticulum cells from the perifollicular mantle towards the red pulp. H & E, × 195.

secondary follicles and minute nests of pyroninophilic cells in the cortex. Involution followed quickly at 8 months when mast cells, macrophages, areas of fibrosis, some scattered pyroninophilic cells and a few plasma cells in the medullary sinuses, were all that remained. At this age the nodes of the conventional NZB mice were distinguished by large active follicles and a medulla composed of hyperplastic reticulum cells and immature plasma cells. Subsequently, reticulum and/or plasma cells contributed to the gross lymphadenopathy characteristic of ageing conventional NZB animals.

Mesenteric lymph nodes. The mesenteric nodes of the germ-free NZB mice were also smaller as compared with conventional NZB animals but, like the spleen rather than the inguinal nodes, they enlarged with age albeit to a limited extent (Fig. 6a). This was mainly

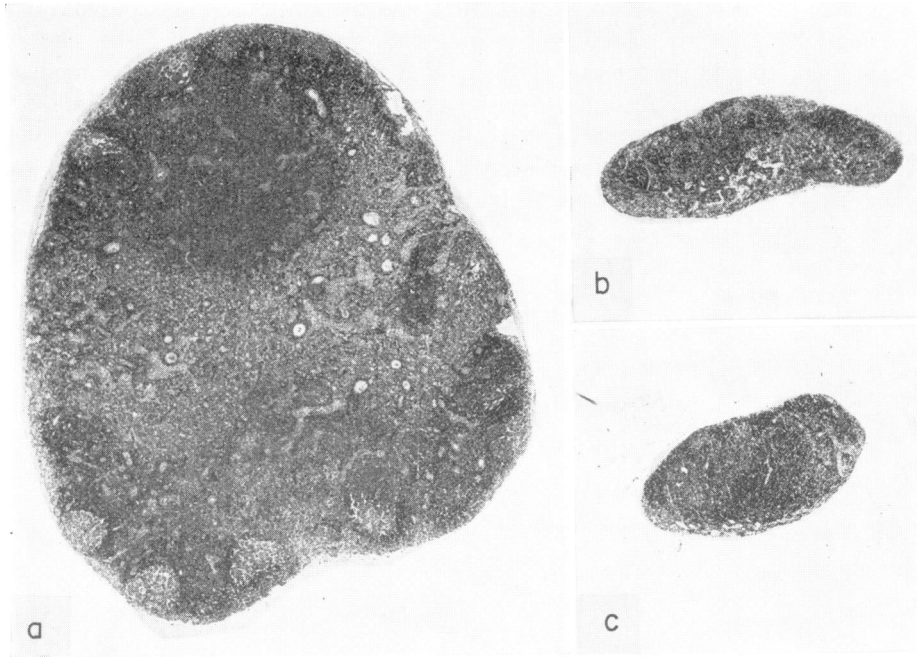

FIG. 5. Inguinal lymph node of: (a) a conventional NZB mouse aged 8 months, (b) a germ-free NZB mouse aged 8 months, and (c) a germ-free CFW mouse aged 8 months. H & E, × 22·5.

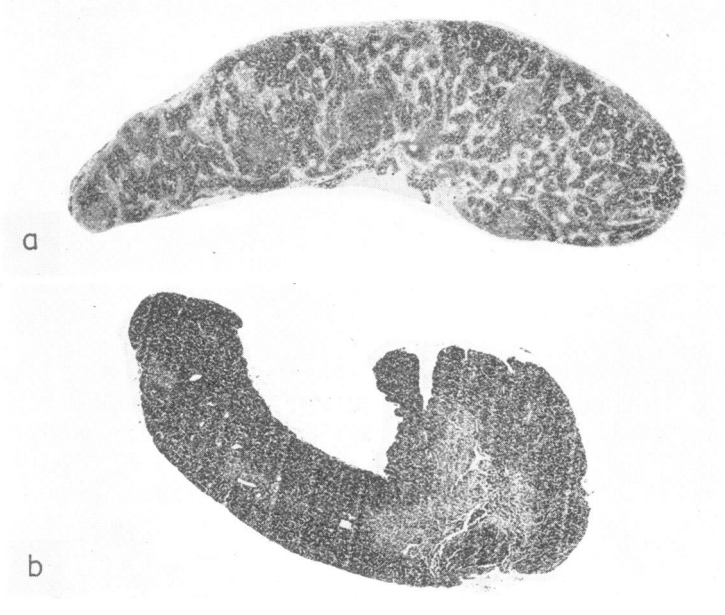

FIG. 6. Mesenteric lymph node of: (a) a germ-free NZB mouse aged 8 months (note lymphocyte depletion in cortex), and (b) a germ-free CFW mouse aged 8 months. H & E, × 22·5.

due to increasing numbers of large pyroninophilic cells arranged in nests in the medullary sinuses (Fig. 7). However, some cortical follicles with germinal centres of strongly pyroninophilic cells were always present and were particularly obvious at 10 months. On the contrary, the cortex of the mesenteric nodes of the germ-free CFW mice was devoid of follicles (Fig. 6b) although it contained pyroninophilic cells.

Thymus. Of all the lymphoid organs, only the thymus was consistently bigger in the germ-free than in the conventional NZB mice 6–10 months old. During this period the cortex was fully populated by lymphocytes which were very active in the subcapsular zone. The medulla was larger, particularly in the germ-free females, and often contained distinct areas of proliferating epithelial cells (Fig. 8b), cords of pyroninophilic cells, many macrophages and cysts full of debris. Medullary germinal centres, which we suspect originated as focal

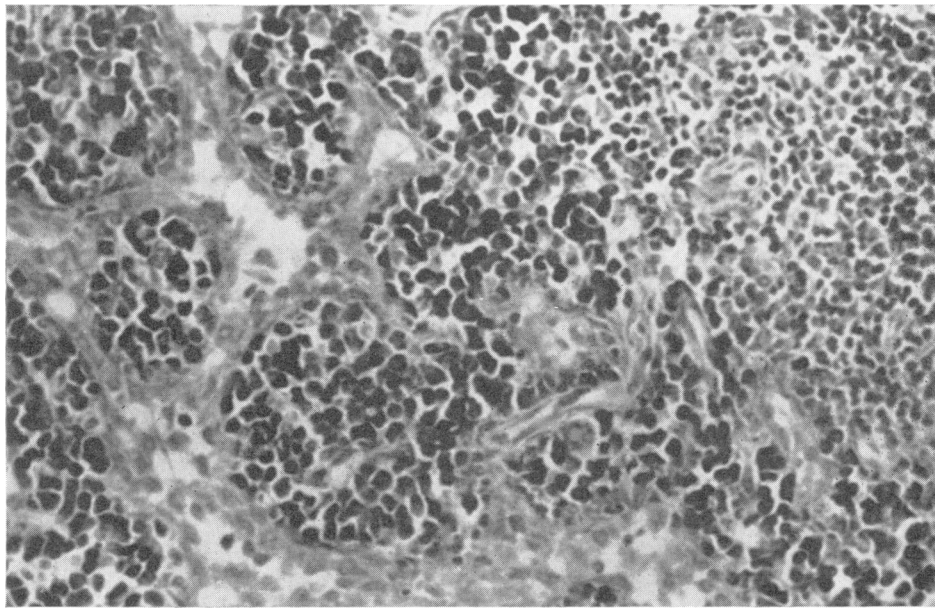

FIG. 7. Nests of pyroninophilic cells in the medullary sinuses of the node shown in Fig. 6(a). Methyl-green–pyronin, × 540.

aggregates of lymphocytes and plasma cells in the perivascular connective tissue between the lobules, were found in three of the thirteen germ-free NZB mice examined. After 10 months the cortex in some of the animals was less defined and showed patchy loss or generalized depletion of lymphocytes, while in others there was cortical inversion (cortex and medulla reversed in dimension). Nevertheless, proliferating epithelial cells and pyroninophilic cells persisted in the medulla which, in the oldest germ-free mice, contained mast cells and macrophages.

Kidneys. The extraordinary, spontaneous glomerulonephritis and lupus nephritis of conventional NZB mice also developed in the kidneys of the germ-free animals. The first lesions appeared rather earlier (at 6 months) in the germ-free NZB mice as crescentic

epithelial proliferations of the glomerular capsule, irregular thickening of the capillary basement membranes and cellular degeneration of the tubules. Lymphocytes and plasma cells clustered around the arteries in the body and pelvis of the kidney. From the age of 8 months the glomeruli enlarged and the lesions became more extensive and were often extreme. The capillaries assumed a characteristic 'wire loop' appearance accompanied by proliferation of the endothelial cells (also of the mesangial cells), fibrinoid thrombi, foci of nuclear disintegration, and moderate to severe sclerosis and hyalinization (Fig. 8a), which gave the tuft a lobulated appearance. Damage to the tubules was indicated by the presence of degenerating cells and PAS-positive casts in their lumina. Sometimes the arteries of the kidney pelvis showed signs of sclerosis. The kidneys of the germ-free CFW mice were normal.

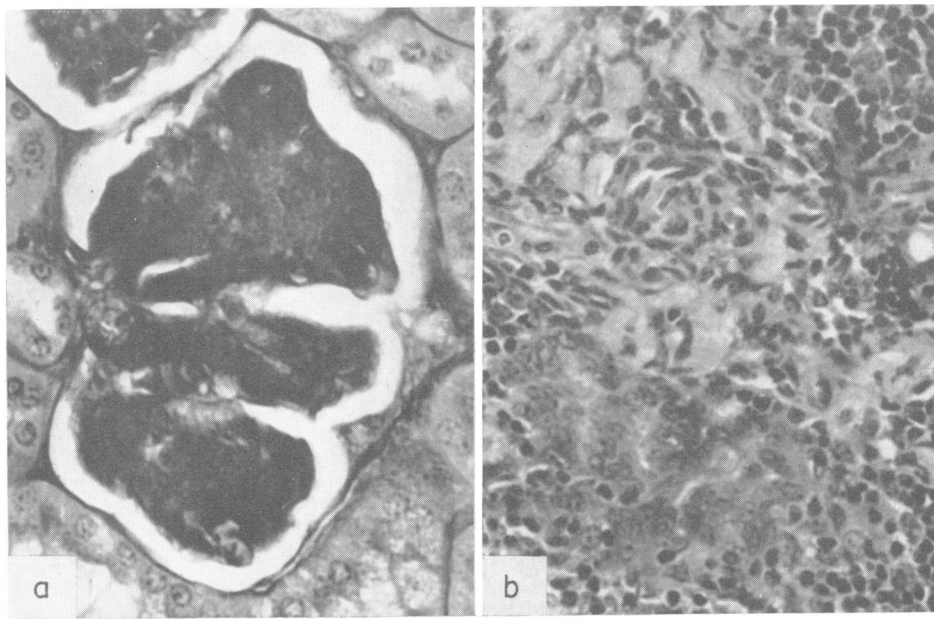

FIG. 8. (a) Kidney glomerulus of a germ-free NZB mouse aged 8 months showing hyalinization and lobulation of the tuft. PAS, × 1800. (b) Thymic medulla of a germ-free NZB donor aged 10 months showing spindle-shaped (above) and polygonal (below) epithelial cells. H & E, × 435.

Lungs. Lesions occasionally seen in the alveolar capillaries of the germ-free NZB mice were reminiscent of, although much less intense than, those present in their renal glomeruli. In addition plasma cells, eosinophils and PAS-positive material sometimes accumulated around the bronchi and arteries and infiltrated the thickened septae. The walls of the large arteries were thickened and, in the older mice, there were clear signs of cellular degeneration.

Gut. A few pyroninophilic cells were noted in the submucosa of the germ-free NZB mice and plasma cells and eosinophils were disseminated through the mucosa. Only minute foci of lymphocytes were found in the submucosa of the germ-free CFW mice.

Pancreas. In some germ-free NZB mice there were small aggregates of lymphocytes close to the Islands of Langerhans.

Liver. Small foci of dying parenchymal cells were the only abnormality detected in the livers of both the germ-free and conventional NZB mice, perhaps rather earlier in the former animals.

Passage of spleen cells (Table 2)

Spleen cell suspensions prepared from three germ-free NZB mice age 10–17 months were passaged by intraperitoneal injection into groups of newborn conventional NZB and BALB/c recipients.

The newborn NZB recipients of inocula from one female germ-free donor (K) aged 10 months developed enlarged spleens weighing 0·7–1·5 g 3–7 months after injection. Older conventional recipients were used in the two subsequent serial passages but they deteriorated very rapidly and were killed with gross splenomegaly only 13–24 days later. Initially, the

TABLE 2. Serial passage of spleen cell suspensions from germ-free NZB donors to conventional NZB and BALB/c recipients

Donor	Pass No.	Strain of recipients	Age of recipients (days)	No. mice with splenomegaly*/ No. mice injected	Mean latent period and range (days)
K: germ-free female NZB age 10 months	1	NZB	1	7/7	139 (103–213)
	2	NZB	13	4/4	13 (–)
	3	NZB	114	8/8	24 (–)
L: germ-free female NZB age 10 months	1a	NZB	1	3/5	314 (245†–351)
	1b	BALB/c	1–2	6/6	395 (342–428)
	2a	NZB	15	5/5	18 (17–19)
	2b	BALB/c	2–5	6/6	24 (21–29)
M: germ-free female NZB age 17 months	1	BALB/c	1–2	4/4	402 (396–426)

* All recipients had generalized reticulum cell neoplasia.

† One mouse only with premature splenomegaly used as donor for the second passage.

results of cell passage from a second 10-month-old germ-free donor (L) seemed disappointing. Some of the NZB recipients died at the same time as untreated controls and only one showed premature splenomegaly at 8 months. After a further passage, however, the tissues of this animal caused splenomegaly and death within 17–19 days of injection into syngeneic recipients. Moreover, allogeneic BALB/c mice which also received the original germ-free material ultimately deteriorated and were killed 11–15 months after injection with spleens weighing 1·3–1·7 g (controls 0·1–0·2 g). Further passage in BALB/c recipients reduced the latent period immediately to 24 days.

The spleens of the BALB/c mice given cell suspensions from the oldest germ-free NZB donor examined (M) became palpable 13 months later and this passage is still in its preliminary stages.

It is important to note that successful cell passage did not precipitate Coombs conversion in any of the conventional recipient NZB mice or induce positive antiglobulin reactions

de novo in the BALB/c recipients, so confirming previous observations (East *et al.*, 1967c; East & Prosser, 1967).

Histologically, all the conventional recipients of series K and L had extensive, often massive, neoplastic proliferations of very large, aberrant, reticulum cells (Fig. 9) in their spleens, liver and lungs. The diffuse distribution of the malignant cells in the spleen and in the sinusoids and portal areas of the liver seemed more obvious in the NZB recipients while, in the BALB/c mice, peribronchial and periarterial accumulations in the lungs were more striking. The inguinal and mesenteric lymph nodes of the first recipients were extremely hyperplastic but in later recipients were frankly neoplastic. The thymus and kidneys were

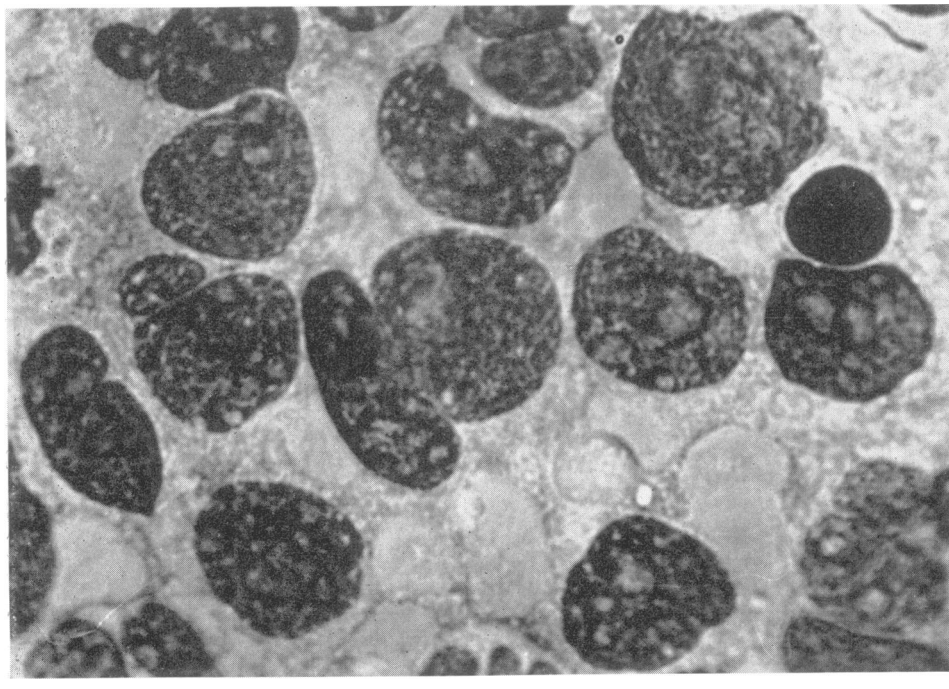

FIG. 9. Malignant reticulum cells in a spleen imprint of a BALB/c recipient given NZB spleen cells at 2 days and killed with gross splenomegaly 342 days later. May–Grüenwald–Giemsa, ×1800.

not involved and no tumours were found at the point of inoculation. The type of malignant cell shown in Fig. 9 is that seen most commonly after passage of conventional NZB material but another form can also occur as described previously (East *et al.*, 1967c; East & Prosser, 1967). This latter type, distinguished by its irregularly clumped nuclear chromatin, comprised the malignant population in the BALB/c recipients of spleen cells from the third germ-free donor (M).

DISCUSSION

Small, inactive spleens and lymph nodes containing only occasional germinal centres and plasma cells, are characteristic of mice maintained in antigenic isolation (Gordon, 1959;

Bauer *et al.*, 1963; Olson & Wostmann, 1966; Dukor, Miller & Sacquet, 1968), and also typified the very few germ-free CFW mice which we examined. However, despite the isolation of NZB mice under identical germ-free conditions, their spleens increased in size and showed a sequence of histological events which, although slightly retarded and not so intense, was similar to that of their conventional NZB counterparts. The first of these events, the development of germinal centres in numerous splenic follicles and the increasing numbers of large pyroninophilic cells in the red pulp, began slowly at 6 months and intensified at 8–10 months during which time the germ-free NZB mice became Coombs positive. It is reasonable to assume, therefore, that this histological picture represented a 'pure' autoimmune reaction to endogenous antigenic stimulation. Moreover, the very local nature of the reaction was emphasized by the fact that the inguinal lymph nodes of these same germ-free NZB mice were almost inactive and involuted very quickly. That the autoimmune response was humoral rather than cell-mediated is supported by reports that conventional NZB mice depleted of lymphocytes by neonatal thymectomy can become Coombs positive (Heyler & Howie, 1963b; East *et al.*, 1967b).

Thymic development proceeded apace in germ-free NZB mice, consistent with the situation described by Bealmear & Wilson, (1966) for germ-free CFW mice. However, the functional significance of the abnormal numbers of pyroninophilic and epithelial cells present in the thymus of both the ageing germ-free and conventional NZB animals, is not yet understood. If these abnormalities reflect in any way the autoimmune activity occurring elsewhere in the body, one wonders why the thymus, but not the inguinal lymph nodes, of the germ-free NZB mice reacted in this manner.

The changes in the mesenteric nodes were also difficult to interpret since pyroninophilic cells were present in germ-free NZB and CFW animals but only the former had some cortical germinal centres. Possibly the mesenteric nodes of both strains were reacting to residual dietary antigen, but the reaction was a little more exaggerated in the NZB mice.

It was very evident that germ-free existence did not preclude the development of severe glomerular lesions in the kidneys of the NZB mice. According to Lambert & Dixon (1968) and McGiven & Ironside (1968) such lesions, which are particularly severe in conventional F_1 (NZB × NZW) hybrids, are not the result of an autoimmune response to abnormal antigens in the basement membranes of the glomerular capillaries, or due to non-specific accumulation of immunoglobulins, but are complexes of nuclear antigens and antinuclear antibody. The similarity of the vascular lesions that occurred not only in the kidney glomeruli but, with decreasing severity, in the spleen, lungs and thymic medulla of the germ-free NZB mice also argued for a systemic aetiology.

Whether abnormality of the target cells or the immunological apparatus is responsible for the autoimmune haemolytic anaemia remains an open question but at least the evidence from the germ-free NZB mice narrows the field by excluding exogenous microbial agents which might cross-react antigenically with target cells. However, the identification of endogenous type 'C' virus particles, morphologically indistinguishable from murine leukaemia virus, in germ-free as well as in conventional NZB mice, immediately suggests an alternative candidate. It is possible that the endogenous virus particles acted as a direct antigenic stimulus and evoked the initial histological changes in the spleens of the germ-free mice. We think this unlikely because the virus particles are known to be particularly numerous in the inguinal lymph nodes of the germ-free NZB mice (Prosser, 1968) which, histologically, were the most inactive of all the lymphoid organs examined. Further, the

virus is probably transmitted via the germ cells and/or placenta (East *et al.*, 1967a) and is certainly present in the embryo and thoughout life (East *et al.*, 1967a; Prosser, 1968) for which reason it need not excite any undue immunological attention from its host. There is no doubt that existing strains of murine leukaemia virus, if deliberately inoculated, can modify both the antigenicity of their target cells and the immunological apparatus of their hosts and so, theoretically, could indirectly precipitate autoimmune reactions. However attractive these theories, which have recently been discussed (East, 1969), there is, to date, no hard experimental proof of viral implication in the complex immunological processes of the NZB mice.

On the other hand, it is an established fact that 'C' type virus is responsible for lymphocytic, erythroid and myeloid leukaemia in conventional mice of many strains, and has been identified in germ-free mice which develop lymphocytic leukaemia either spontaneously or after X-irradiation (Pollard & Matsuzawa, 1964; Pollard, Kajima & Teah, 1965). There are, therefore, considerable precedents for assuming a link between the virus particles and the reticulum cell neoplasia which develops in ageing conventional NZB mice and which we have now demonstrated in their germ-free counterparts histologically and by cell passage. Our studies showed that the malignant reticulum cells proliferated in the immunologically active spleens of the germ-free NZB mice but not in their immunologically inactive inguinal lymph nodes. These malignant changes in the spleen were, moreover, the final phase of events which began with a sudden burst of immunological activity several months earlier. Within the spleen the reticulum cells appeared to originate in, and disseminate from, the active splenic follicles. We feel these facts justify speculation that the continuous stimulation of autoimmune activity was a pre-requisite either for the supply of suitable target cells available to, or for the proliferation of cells already transformed by, the ever-present virus. But the nature of the trigger or triggers to the autoimmunity remains unknown.

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