Clin. exp. Immunol. (1968) **3**, 657–663.

ANTINUCLEAR FACTOR IN NZB/NZW MICE: INCIDENCE AND *IN VITRO* EFFECTS

A. R. McGIVEN AND T. GHOSE

Department of Pathology, Monash University, Melbourne, Australia

(*Received* 26 *February* 1968)

SUMMARY

After the age of 6 months, NZB/NZW mice develop almost 100% incidence of serum antinuclear factor (ANF) which is located in the IgG immunoglobulin fraction, binds guinea-pig complement and gives homogeneous, membranous and speckled immunofluorescent nuclear staining patterns. No specific *in vitro* cytotoxic activity against homologous kidney cells was detected in serum from these mice, but their lymphoid cells clustered around a proportion of kidney cells which subsequently degenerated.

INTRODUCTION

The F_1 hybrid NZB/NZW mice develop a form of glomerulonephritis associated with positive lupus erythematosus (LE) cell tests (Helyer & Howie, 1963; Burnet & Holmes, 1965; Miyasato, Manaligod & Pollak, 1967). This study was undertaken to determine the incidence and character of antinuclear factor (ANF) in NZB/NZW mice and to assess whether serum or lymphoid cells from NZB/NZW mice with the factor possessed *in vitro* cytotoxic activity.

MATERIALS AND METHODS

Serum

Blood was collected from the heart or tail veins of ninety-nine male and ninety-four female NZB/NZW mice with ages ranging from 1 to 11 months.

Serum fractionation

Several strongly reacting sera were separated into 7S and 19S fractions on a Sephadex G-200 column.

Correspondence: Dr A. R. McGiven, Department of Pathology, Monash University Medical School, Alfred Hospital, Prahran, Victoria, Australia.

Immunofluorescent staining

Tissue blocks were snap-frozen in an isopentane–liquid nitrogen mixture at −160°C and sections 4–6 μ thick were cut in a cryostat at −20°C. Rat liver sections were immersed in 95% ethanol at 4°C for 1 min. Human thyroid and mouse kidney sections were used unfixed. Human blood films were air-dried and immersed in absolute ethanol for 10 sec. All preparations were air-dried at 2°C for at least 2 hr before use.

Mouse sera were tested for antinuclear activity by covering sections or blood films with the test sera for 30 min, washing twice for 5 min in phosphate-buffered saline (0·01 M-phosphate, pH 7·1) then covering with fluorescein-conjugated rabbit anti-mouse-globulin (Nutritional Biochemicals Corporation) for 30 min, washing twice and mounting in buffered glycerol. The preparations were examined by darkground ultraviolet fluorescence microscopy.

Fixation of guinea-pig complement

To demonstrate binding of guinea-pig complement to the nucleus–antinuclear factor complex, the tissue sections or blood films, after first being covered with mouse serum for 30 min and washed twice in the phosphate buffer, were covered with a drop of fresh guinea-pig serum diluted 1 : 20 with complement-fixing diluent containing calcium and magnesium (Oxo Ltd) and incubated in a moist chamber at 37°C for 30 min. Excess guinea-pig serum was rinsed off the sections which received two further 5 min washings. They were then treated for 30 min with a fluorescein-conjugated anti-guinea-pig complement serum prepared in rabbits (Klein & Burkholder, 1960). Sections were mounted after two final washes. Controls included the use of mouse serum which did not contain antinuclear factor, and the inactivation of complement in guinea-pig serum by heating at 56°C for 30 min.

Tissue culture

Fresh kidneys and thyroids obtained from NZB/NZW hybrid and Balb/c mice, less than 1 week old, were collected and diced into 2-mm cubes. These fragments were washed in several changes of Hanks's balanced salt solution containing 200 units of penicillin and 100 units of streptomycin per millilitre and were subsequently grown as explant cultures on 'flying coverslips' in roller tubes containing medium 199 supplemented with 20% foetal calf serum. After about 5–7 days most of the explants formed a uniform monolayer of cells around the parent tissue fragment. Irrespective of whether the explants were derived from kidney or thyroid, the monolayers consisted either of pure epithelium, pure fibroblasts or a mixture of both cell types. Suspensions of lymphocytes were prepared from lymph nodes and spleen of young (less than 1 week old) and adult NZB/NZW and Balb/c mice by teasing small pieces of lymph nodes and spleen in calcium- and magnesium-free balanced salt solution containing 0·02% ethylene diaminotetracetic acid (EDTA). Dye exclusion tests revealed at least 95% of viable lymphocytes in these suspensions.

To study the cytotoxic activity of adult NZB/NZW mouse sera, the coverslips incorporating the above mentioned cultures were transferred to Rose chambers containing Hanks's balanced salt solution and a few drops of 0·5% sterile solution of trypan blue in normal saline. The cultures were examined with an ordinary microscope to determine the proportion of trypan blue-permeable cells and with an inverted phase-contrast microscope to examine the morphology of the monolayer cells. Immediately after the microscopic examinations enough adult NZB/NZW mouse serum was introduced into the Rose chambers to give a

50% concentration of serum. Monolayer cultures of epithelium and fibroblasts from kidney and thyroid of young NZB/NZW and Balb/c mice were exposed in this way to sera from young and adult NZB/NZW and Balb/c mice and a guinea-pig anti-mouse serum of proven cytotoxicity to mouse cells. Complement was added to half the preparations with each type of tissue. At least six different cultures of each type of cell were tested with every serum and each experiment in its entirety was repeated twice. The preparations were examined by brightground and phase contrast microscopy for evidence of cytotoxic activity $\frac{1}{2}$, 1, 2, 4, 24 and 48 hr after the exposure of the cultures to the test sera.

To study any cell injury caused by cell suspensions from lymph nodes and spleen, coverslips containing each type of established monolayer were transferred to Rose chambers filled with either $0{\cdot}5 \times 10^7$ or 10^9 viable lymphocytes suspended in Hanks's balanced salt solution and a few drops of 0·5% sterile trypan blue. Complement was added to a few chambers containing cultures of each type. The preparations were examined microscopically as before after 1, 2, 4, 18 and 24 hr and then once every day for 6 days, after which at the completion of the experiments they were fixed in 10% neutral formalin and stained with haematoxylin and eosin.

RESULTS

Immunofluorescence

The incidence of antinuclear factor in NZB/NZW mice is given in Table 1. Antinuclear factor was not detected until the age of about 6 months and reached an incidence of almost 100% in females by 7 months and in males by 10 months. The fluorescent patterns of nuclear staining on tissue sections included homogeneous, membranous and speckled and usually more than one pattern was present. Nucleolar staining was not detected. On blood films, nuclear staining was homogeneous (Fig. 1) often with a brightly staining nuclear membrane. Nuclei of polymorphonuclear leucocytes commonly stained more strongly than did nuclei of mononuclear cells present in the blood film. The homogeneous pattern of staining could be prevented by absorption of the mouse sera with nucleohistone or pretreatment of sections or blood films with deoxyribonuclease. Speckled staining was inhibited by washing sections in 0·15 M-NaCl for 2 hr before attempting staining.

Antinuclear factors responsible for homogeneous and speckled patterns were each able to bind guinea-pig complement (Fig. 2). Antinuclear activity was confined to the IgG γ-globulin fraction.

No anti-renal activity against the cytoplasm of NZB/NZW renal tubular cells was detected in NZB/NZW mouse sera by immunofluorescence.

Tissue culture

Most of the adult NZB/NZW mouse sera as well as the sera from young NZB/NZW and Balb/c mice failed to show any cytotoxic activity on any of the monolayer cultures used in these experiments. An occasional adult NZB/NZW mouse serum together with complement rendered about 15–20% cells of the exposed monolayers permeable to trypan blue in about 30 min. But an occasional Balb/c mouse serum also revealed a similar cytotoxic activity and in all cases the cytotoxicity was similar irrespective of the nature and origin of the exposed monolayer cells. All the adult NZB/NZW mouse sera used in these experiments contained antinuclear activity. Sera from young NZB/NZW and Balb/c mice did not possess any antinuclear activity.

In allogeneic combinations, i.e. when NZB/NZW kidney and thyroid cells were exposed to Balb/c lymphocytes or when Balb/c kidney and thyroid cells were exposed to NZB/NZW lymphocytes, clustering lymphocytes around the individual cells in the monolayers could be seen from about 24 hr after the start of the experiment. Only about 10–20% of cells, especially

TABLE 1. Incidence of antinuclear factor in NZB/NZW mice

Age (months)	Male		Female	
	Positive	Negative	Positive	Negative
1	0	8	0	11
2	0	14	0	14
3	0	9	0	10
4	0	8	0	6
5	0	4	0	6
6	0	16	9	3
7	3	6	23	1
8	8	7	6	0
9	1	1	4	0
10	6	0	1	0
11	6	2		
Total	24	75	43	51

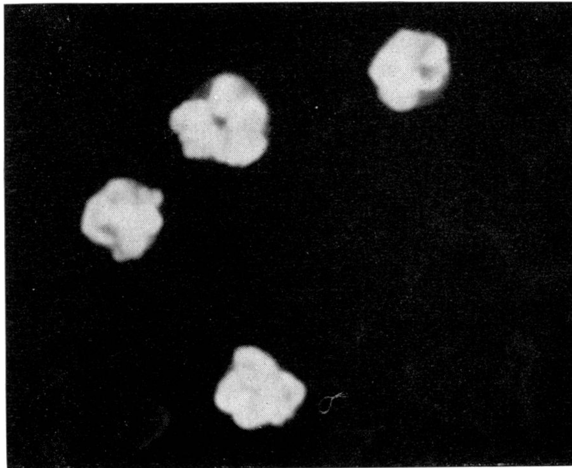

FIG. 1. Human blood film showing homogeneous nuclear staining of leucocytes by serum from NZB/NZW mouse. × 1000.

those at the periphery of the monolayer sheets, revealed aggregation of lymphocytes around them.

When monolayers of Balb/c kidney and thyroid origin were exposed to Balb/c lymphocytes, only an occasional cell (less than 1%) showed a few lymphocytes aggregated on their surface.

Lymphocytes from adult NZB/NZW mice on the other hand clustered around 15–20% of NZB/NZW kidney cells in monolayer (Fig. 3). This clustering of lymphocytes occurred to

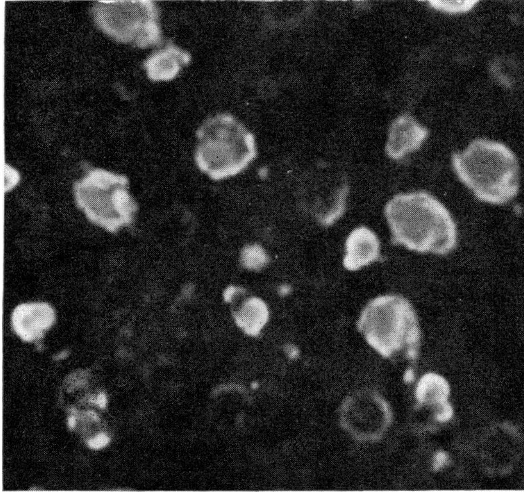

FIG. 2. Section of rat liver showing binding of guinea-pig complement to nucleus–mouse ANF complex. ×600.

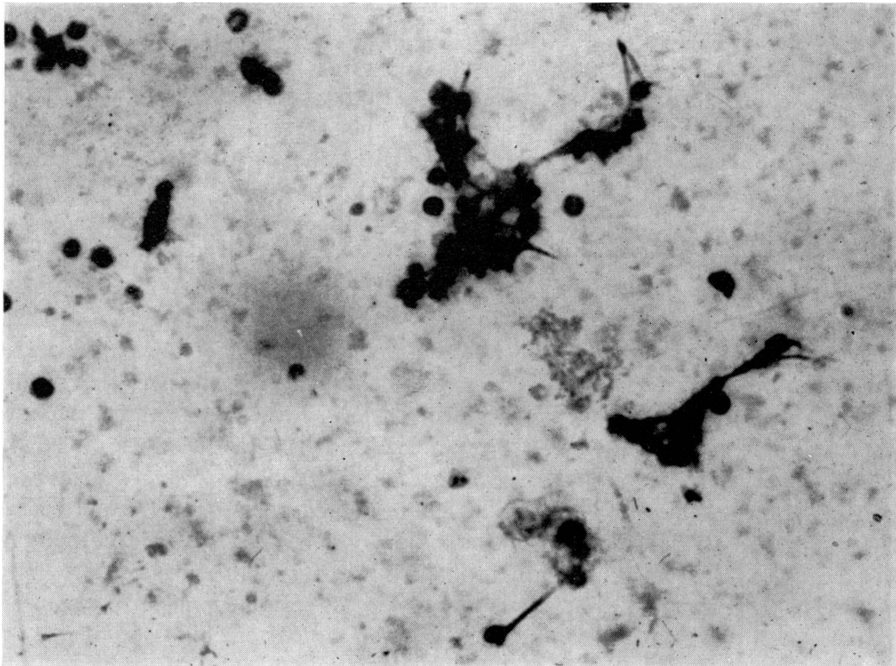

FIG. 3. Neonatal NZB/NZW mouse kidney cells grown as monolayer cultures and exposed to adult NZB/NZW mouse lymphocytes for 24 hr. Note the clustering of lymphocytes on the surface of the kidney cells. H & E. ×160.

the same extent irrespective of whether the kidney monolayer cells were epithelial or fibroblastic. Lymphocytes from old NZB/NZW mice also clustered around fibroblasts derived from young NZB/NZW thyroid explants, though only around 5–10% of the thyroid cells. The lymphocytes which clustered around the monolayer cells did not show any morphological change apart from the general degeneration occurring in the cultures attributable to advancing age or to manipulations.

It was very difficult to detect any morphological change in the monolayer cells around which lymphocytes clustered because of the density of the lymphocyte aggregates. Although in the stained preparations many of these monolayer cells showed nuclear disintegration and cytoplasmic eosinophilia, it cannot definitely be stated whether the lymphocytes were causing cell injury or whether they were preferentially adhering to cells already injured by the tissue culture environment. Observation by phase-contrast microscopy suggested that most of the lymphocytes aggregated on the cell surface and that the phenomenon observed was not emperipolesis. Lymphocytes clustered around more monolayer cells when the Rose chambers contained 10^9 lymphocytes. Complement did not appear to have any effect on the lymphocytic clustering.

DISCUSSION

The occurrence of antinuclear factors in the sera of NZB/NZW mice by the age of 6 months is consistent with the previous reports of positive LE cell tests. The nuclear staining patterns resemble those described by Beck (1963) in human sera from patients with systemic lupus erythematosus (SLE): the homogeneous pattern is given by an antibody reacting with nucleohistone and the speckled pattern by an antibody reacting against a saline soluble antigen. The absence of antinuclear activity in the macroglobulin fraction is different from the findings in human subjects with SLE, where the IgM fraction may give a speckled pattern of nuclear staining (Bonomo, Tursi & Dammacco, 1965).

In tissue culture, the cytodestructive effects of lymphocytes aggregated on target cells were not as powerful as those described by Trayanova, Sura & Svet-Moldavsky (1966) with lymphocytes from SLE patients or by Govaerts (1960) in kidney graft rejection studies. Holm (1966), in his study of the interaction between radio-labelled target kidney cells and lymphocytes from rats with experimental autoimmune nephritis, has shown a fair correlation between surface aggregation of lymphocytes and damage to target cells. The results presented here, therefore, correspond fairly well to the observation in the human disease SLE that sera containing ANF are not cytotoxic (Ward, Cloud & Turner, 1964), though lymphoid cells from these patients are capable of causing cell destruction *in vitro* (Trayanova *et al.*, 1966). Prominent collections of lymphocytes can be seen in the kidneys of adult NZB/NZW mice extending along blood vessels from the hilum to the cortex, and also in the lungs and intestine (de Vries & Hijmans, 1967). It is possible that these collections of lymphoid cells may be causally associated with at least some of the tissue damage in these organs.

Although the antinuclear antibodies in NZB/NZW mouse sera bind complement during their reaction with nuclear antigens, they have not shown any cytotoxic activity. Impermeability of living cells to globulins and the consequent inaccessibility of the nuclear antigens may be one of the factors to explain this. On the other hand, antinuclear antibodies, though not cytotoxic by themselves, could combine *in vivo* with nuclear antigens from dead or

injured cells to form circulating immune complexes which might initiate the glomerular lesions in these mice (McGiven & Ironside, 1968).

ACKNOWLEDGMENTS

We wish to thank Professor R. C. Nairn for helpful advice, Dr P. N. J. Ironside for serum fractionation, Dr P. M. Burkholder for conjugated anti-guinea-pig-complement, Miss L. Canobio, Mrs M. Cerini and Miss C. MacNab for technical assistance. The work was supported by grants from the Australian Research Grants Committee, the National Health and Medical Research Council and the Anti-Cancer Council of Victoria.

REFERENCES

Beck, J.S. (1963) Auto-antibodies to cell nuclei. *Scot. med. J.* **8**, 373.

Bonomo, L., Tursi, A. & Dammacco, F. (1965) Characterization of antibodies producing the homogeneous and the speckled fluorescence patterns of cell nuclei. *J. Lab. clin. Med.* **66**, 42.

Burnet, F.M. & Holmes, M.C. (1965) The natural history of the NZB/NZW F_1 hybrid mouse: a laboratory model of systemic lupus erythematosus. *Aust. Ann. Med.* **14**, 185.

Govaerts, A. (1960) Cellular antibodies in kidney homotransplantation. *J. Immunol.* **85**, 516.

Helyer, B.J. & Howie, J.B. (1963) Renal disease associated with positive lupus erythematosus tests in a crossbred strain of mice. *Nature* (*Lond.*), **197**, 197.

Holm, G. (1966) *In vitro* cytotoxic effects of lymphoid cells from rats with experimental autoimmune nephrosis. *Clin. exp. Immunol.* **1**, 45.

Klein, P.G. & Burkholder, P.M. (1960) Studies on the antigenic properties of complement. I. Demonstration of agglutinating antibodies against guinea-pig complement fixed on sensitized sheep erythrocytes. *J. exp. Med.* **111**, 93.

McGiven, A.R. & Ironside, P.N.J. (1968) Elution of antinuclear factor from renal lesions of NZB/NZW mice. *Clin. exp. Immunol.* **3**, 665.

Miyasato, F., Manaligod, J.R. & Pollak, V.E. (1967) Auto-immune disease in NZB and NZB-NZW F_1 mice. *Arch. Path.* **83**, 20.

Trayanova, T.G., Sura, V.V. & Svet-Moldavsky, G.J. (1966) Destruction of human cells in tissue culture by lymphocytes from patients with systemic lupus erythematosus. *Lancet*, **i**, 452.

Vries, M.J. de & Hijmans, W. (1967) Pathological changes of thymic epithelial cells and autoimmune disease in NZB, NZW and (NZB × NZW) F_1 mice. *Immunology*, **12**, 179.

Ward, J.R., Cloud, R.S. & Turner, L.M. (1964) Noncytotoxicity of 'nuclear antibodies' from lupus erythematosus sera in tissue culture. *Ann. rheum. Dis.* **23**, 381.