Immunology, 1974, **26**, 893.

The Long-Term Antibody Response of New Zealand Black Mice to Sheep Red Blood Cells

ELISABETH A. S. ELKERBOUT* AND W. HIJMANS

Institute for Experimental Gerontology, Organization for Health Research TNO, 151 Lange Kleiweg, Rijswijk (Z.H.), The Netherlands

(*Received 23rd May* 1973; *accepted for publication 2nd November* 1973)

Summary. Long-term IgM and IgG responses to sheep red blood cells (SRBC) were determined in New Zealand Black (NZB) mice, using the plaque technique. As compared with other strains a prolonged spleen plaque-forming cell (PFC) response was found in young and old NZB mice. This phenomenon was found in the primary as well as in the secondary response and concerned IgM as well as IgG production. These observations are discussed in relation to the production of IgG antibodies of a low avidity.

INTRODUCTION

Since the first report on haemolytic anaemia in New Zealand Black (NZB) mice (Bielschowsky, Helyer and Howie, 1959), these animals have been studied extensively as a model of autoimmune disease. Several pathways leading to the abnormal immune reactions in NZB mice have been suggested. These include changes in the immune mechanisms (Burnet and Holmes, 1962; Staples, Steinberg and Talal, 1970) or the presence of modified target cells (Holborow and Denman, 1967; Braverman and Slesinski, 1968). According to Mellors, Shirai, Aoki, Huebner and Krawczynski (1971) a leukaemia-like virus is directly responsible for the disease. Talal, Steinberg, Jacobs, Chused and Gazdar (1971) suggest that viruses may act as antigenic stimuli for a genetically hyper-responsive antibody-producing system.

The immune response to exogenous antigens has also been studied extensively, especially with sheep red blood cells (SRBC). Disturbances such as early maturation (Evans, Williamson and Irvine, 1968; Playfair, 1968) hyper-responsiveness in animals aged 6–14 weeks (Morton and Siegel, 1969; Cerottini, Lambert and Dixon, 1969; Baum, 1969) and hypo-responsiveness in old animals (Diener, 1966; Morton and Siegel, 1969) have been described.

We have made long-term observations of the immune response in NZB mice after stimulation with SRBC. Antibody response was studied up to 3 months in the primary response and up to 1 month in the secondary response.

MATERIALS AND METHODS

Animals

The NZB mice have been bred for nine to twelve generations in our animal quarters

* Present address: Department of Radiotherapy, University Hospital Leiden, Leiden, The Netherlands.

since being kindly provided in 1963 as generation 54 by Mr W. Hall, Animal Department, University of Otago School, Dunedin. NZW animals were received from the same source in their twentieth generation. BALB/c mice, A/J mice, and C57Bl mice from our breeding colony were used as controls. All animals were maintained by brother–sister mating. The animals were fed commercial pelleted food with free access to tap water.

Approximately equal numbers of male and female mice were used in the experimental groups. The immune response was studied at different ages, with special emphasis on the response in the older age groups.

Direct Coombs' test

The Coombs' serum used was a heat-inactivated rabbit anti-mouse immunoglobulin serum. It had been absorbed with twelve times washed red cells from Swiss mice (de Vries and Hijmans, 1967). Serum dilutions of 1:5 and 1:50 gave the best results. Tail blood was collected in phosphate-buffered saline (PBS) and the red cells were washed three times. One drop of a 4% suspension was mixed with an equal volume of antiserum diluted 1:50. Negative tests at the 1:50 dilution were repeated with antiserum diluted 1:5. The tests were read on a white earthenware plate with incident light.

Antigen challenge

Except when stated otherwise, a standard dose of 4×10^8 SRBC was injected in the tail vein in a volume of 0·2 ml. The fresh SRBC had been washed three times in PBS.

Preparation of developing antiserum

Mouse ascitic fluid was obtained according to the method described by Munoz (1957) with the modification that the animals were injected with Freund's incomplete adjuvant. Six millilitres of the fluid was applied to a DEAE–cellulose column (50 × 1·2 cm) and the fractions containing the first peak which had been eluted with a 0·01 M phosphate buffer of pH 8·0, were pooled and concentrated to 2 ml by ultrafiltration. Only the one line—corresponding to IgG—was demonstrated in this preparation by immunoelectrophoresis, when using an antiserum against mouse total serum proteins. A rabbit was immunized with 6 mg of the IgG preparation combined with Freund's complete adjuvant in the foot pads, intracutaneously, and intramusculary in multiple sites. Already, 3 weeks after immunization, the serum of the animal gave a strong single precipitin line with mouse IgG in the immunodiffusion systems. No activity against IgM was detected when the antiserum was tested against normal mouse serum or against serum containing an IgM paraprotein (Hijmans, Radema, Es, Feltkamp, Loghem and Schaap, 1969).

Heat-inactivated antiserum was absorbed with washed mouse and sheep red blood cells. Finally it was titrated in the plaque test system according to Dresser and Wortis (1967) with optimal results at a dilution of 1:480.

Preparation of complement

Pooled guinea-pig serum was used as a source of complement. Absorption was carried out by incubating two volumes of serum with one volume of washed mouse red blood cells and one volume of washed SRBC for 45 minutes at 4°. Immediately after absorption the serum was frozen and stored at −70°. To determine the optimal concentration for every pool, different volumes of guinea-pig serum were added to a standard spleen cell suspension in the direct plaque-forming cell test. There were minor differences between the different batches and usually 0·03 ml was found to be optimal.

Plaque formation

Haemolytic plaque-forming cells (PFC) were assayed by a modification of Jerne's technique (Jerne, Nordin and Henry, 1963), using liquid medium (Cunningham, 1965) and glass chambers according to the method of Montgomery Smith (1956).

The main advantages of this technique are: increased sensitivity (Cunningham and Szenberg, 1968); under the light microscope pseudo-plaques are readily recognized; only small amounts of complement and developing serum are required; this method is less time consuming than the agar plaque technique.

Preparation of lymphoid cell suspension

The animals were killed by cervical dislocation. The spleen was removed immediately and put into a disposable Petri dish, containing ice-cold Medium 199. All manipulations were carried out at 4°. A bottle cooler proved to be highly convenient for this purpose. With a glass spatula, each spleen was gently minced through six layers of washed nylon tissue into Medium 199 supplemented with 5 per cent heat-inactivated calf serum. Using calibrated tubes, the volume of the suspension was noted. The content of the tube was mixed by repeated aspiration. One millilitre of the suspension was pipetted into each of two test tubes. In one suspension viable cells were counted immediately in a haemocytometer using dye exclusion (0·2% Trypan Blue in Medium 199 supplemented with 5% calf serum). Spleen cells in the other sample were washed by centrifugation at 1500 r.p.m. for 10 minutes at 4°. The cells were resuspended in 1 ml Medium 199 with calf serum and counted. Unwashed cells were used for a direct PFC test; a direct test and duplicate indirect PFC tests were performed with cells from the washed suspension.

Depending on the interval after immunization, the required suspensions were made up so that 0·1 ml contained 10^5–10^6 viable spleen cells.

Preparation of SRBC suspension

The SRBC to be used as target cells were allowed to 'age' under sterile conditions for at least 7 days. Before the test the cells were washed three times in PBS and resuspended in Medium 199 supplemented with calf serum. The cells were counted and the cell concentration was adjusted so that 0·1 ml contained 5×10^7 SRBC.

Preparation of glass chambers

Coverslips were carefully cleaned with a detergent and then rinsed in running tap water for 24 hours. Glass slides and cleaned coverslips were kept in methanol.

The chambers are made according to the following procedure. On a glass slide (76 × 26 mm), which was placed in a holder, a piece of plastic (76 × 13 × 0·1 mm) and then a coverslip (40 × 24 mm) is laid. The long side of the set consisting of slide, plastic and coverslip was fixed with a clip and then the other long side was dipped in melted paraffin. When the paraffin was hardened, the plastic was removed with a pair of tweezers and the second long side was dipped into the melted paraffin.

Direct PFC test

Consecutively, 0·03 ml complement, 0·1 ml spleen cell suspension, and 0·1 ml SRBC were pipetted into an agglutination tube. The mixture was warmed to 37° for a few seconds. Using a Pasteur capillary pipette, this amount was sufficient to fill three chambers.

Filled chambers were sealed with heated vaseline. After 1 hour of incubation at 37° the plaques were counted under a dissecting microscope.

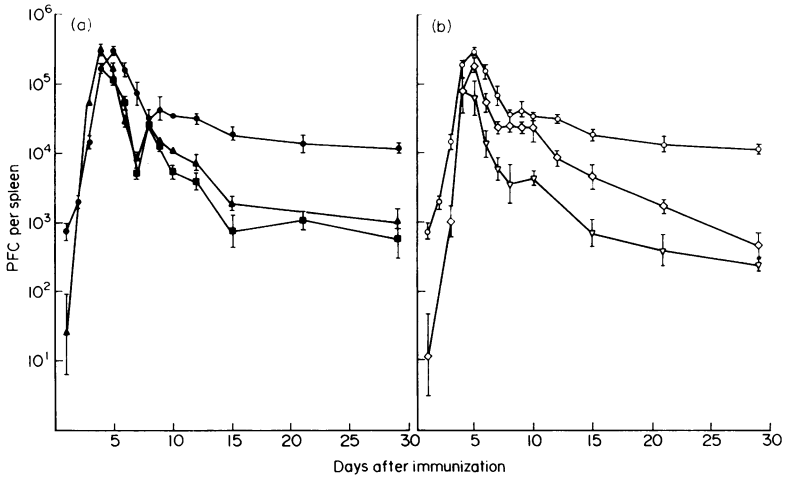

FIG. 1. (a) IgM PFC response following antigen challenge with 4×10^8 SRBC in (●) NZB, (▲) BALB/c and (■) A/J mice, aged 9 months. The values shown are the logarithmic means ± 1 s.e. (b) IgM PFC response following antigen challenge with 4×10^8 SRBC in (○) NZB, (◇) C57Bl and (▽) NZW mice, aged 9 months. The values shown are the logarithmic means ± 1 s.e.

Indirect PFC test

Consecutively, 0·01 ml of optimally diluted rabbit anti-mouse IgG serum, 0·03 ml complement, 0·1 ml spleen cell suspension, and 0·1 ml SRBC were pipetted into an agglutination tube. The cells were tested as described for the direct PFC test. As the developing serum did not inhibit IgM plaque-forming cells, the number of IgG PFC was obtained by subtracting 'direct' plaques from 'indirect' plaques. PFC were measured in individual spleens. The results are expressed as the mean value of five to ten spleens. Because of the wide range in the numbers of plaques within each test series, the exact data together with the standard deviations are given per spleen and per 10^6 spleen cells. For the evaluation of the results it seems worthwhile to mention that the results were not obtained chronologically but in an 'at random' fashion.

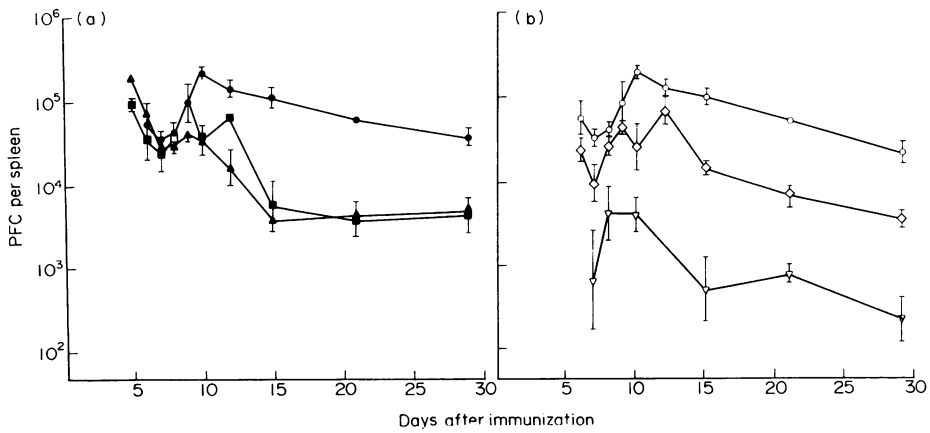

FIG. 2. (a) IgG PFC response following antigen challenge with 4×10^8 SRBC in (●) NZB, (▲) BALB/c and (■) A/J mice, aged 9 months. The values shown are the logarithmic means ± 1 s.e. (b) IgG PFC response following antigen challenge with 4×10^8 SRBC in (○) NZB, (◇) C57Bl and (▽) NZW mice, aged 9 months. The values shown are the logarithmic means ± 1 s.e.

RESULTS

PRIMARY RESPONSE

IgM PFC response in NZB mice and control strains, aged 9 months

Peak response occurred 4 days after immunization in BALB/c, A/J, and NZW mice, and 5 days after antigen challenge in NZB and C57Bl mice (Fig. 1a, b). The highest value was found in BALB/c mice. In NZB mice the decreasing phase of the response was much less steep than in control strains, resulting in a ten to forty times higher number of PFC 1 month after immunization. Three months after immunization 2×10^3 IgM PFC were still detectable in NZB spleens.

TABLE 1

IgM AND IgG PFC RESPONSE PER SPLEEN IN NZB AND BALB/c MICE, AGED 5 MONTHS, AFTER ANTIGEN CHALLENGE WITH 4×10^8 SRBC

Days after immunization	IgM PFC (Logarithmic mean ± 1 s.e.)		IgG PFC (Logarithmic mean ± 1 s.e.)	
	NZB	BALB/c	NZB	BALB/c
4	5·96±0·03	5·88±0·05	0	0
5	5·79±0·09	5·15±0·03	0	5·70±0·08
21	3·90±0·13	3·50±0·04	4·78±0·09	3·96±0·14

TABLE 2

IgM AND IgG PFC RESPONSE PER SPLEEN IN 9-MONTH-OLD NZB AND BALB/c MICE AFTER ANTIGEN CHALLENGE WITH 4×10^8 OR 4×10^9 SRBC

Days after immunization	Antigen dose	IgM PFC (Logarithmic mean ± 1 s.e.)		IgG PFC (Logarithmic mean ± 1 s.e.)	
		NZB	BALB/c	NZB	BALB/c
4	4×10^8 SRBC	5·25±0·08	5·51±0·06	0	0
4	4×10^9 SRBC	5·46±0·14	5·34±0·14	0	0
5	4×10^8 SRBC	5·48±0·06	5·19±0·09	0	5·26±0·08
5	4×10^9 SRBC	5·72±0·05	5·34±0·08	0	5·57±0·11
29	4×10^8 SRBC	4·06±0·06	3·02±0·20	4·39±0·12	3·67±0·11
29	4×10^9 SRBC	4·47±0·12	3·19±0·06	4·88±0·16	4·11±0·15

IgG PFC response in NZB mice and control strains, aged 9 months

IgG PFC in NZB, BALB/c, A/J, C57Bl and NZW mice were first detectable respectively 6, 5, 5, 6 and 7 days after immunization (Fig. 2a,b). Thereafter, the IgG curve first shows an irregular course in all strains. Between the 12th and the 15th day after immunization, a decrease of 17 per cent in the number of PFC per spleen occurred in the NZB mice, whereas in BALB/c, A/J, and C57Bl mice this decrease amounted to respectively 76, 91 and 78 per cent. NZW mice showed a low IgG PFC response 1 month after immunization, comparable to the IgM PFC response.

IgM and IgG PFC response in NZB and BALB/c mice, aged 5 months

In order to determine if a higher response was also present in younger NZB mice, 5-month-old animals were challenged (Table 1). In contrast to 9-month-old animals, 5-month-old NZB mice showed an IgM peak response 4 days after immunization. Higher IgM and markedly higher IgG PFC values were found in NZB mice 3 weeks after immunization in comparison to the values found in BALB/c mice of the same age.

IgM and IgG PFC response in NZB and BALB/c mice aged 9 months, after immunization with 2×10^9 *SRBC*

A number of animals were challenged with a higher antigen dose in order to determine if the plaque-forming capacity in the spleen was saturated (Table 2). In both strains there existed a slight increase of IgM and IgG PFC values after stimulation with a higher antigen dose.

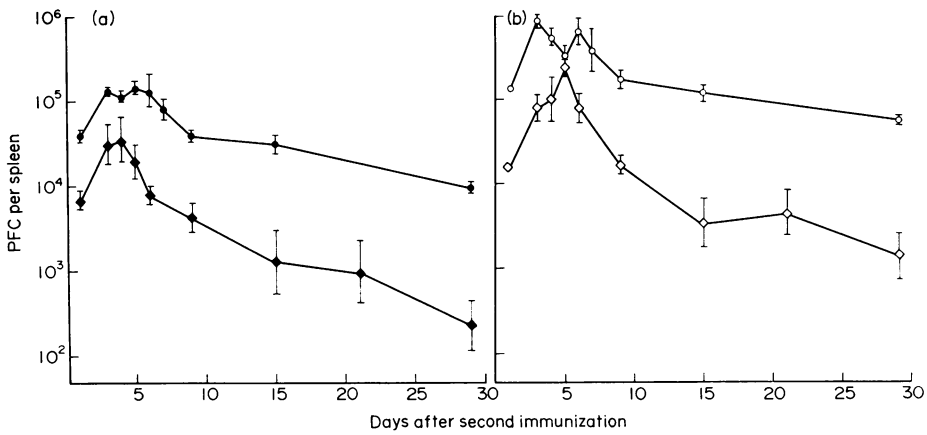

FIG. 3. (a) IgM PFC response following a second injection with 4×10^8 SRBC in (●) NZB and (◆) C57Bl mice, aged 9 months. The first injection was given 21 days before the booster dose. The values shown are the logarithmic means ±1 s.e.
(b) IgG PFC response following a second injection with 4×10^8 SRBC in (○) NZB and (◇) C57Bl mice, aged 9 months. The first injection was given 21 days before the booster dose. The values shown are the logarithmic means ±1 s.e.

SECONDARY RESPONSE

Twenty-one days after immunization, a booster dose of 4×10^8 SRBC was administered. At all days tested NZB mice showed a higher IgM and IgG PFC secondary response than C57Bl mice (Fig. 3a,b).

A prolonged primary and secondary response was also observed if the results were expressed in PFC per 10^6 spleen cells.

BACKGROUND

In order to establish the number of background PFC, twenty-two NZB males and thirteen NZB females were tested. As controls A/J, C57Bl and BALB/c mice were used.

The results derived from these thirty-three normal mice have been grouped together (Fig. 4). The NZB mice showed a higher number of background PFC than the controls. Background PFC in NZB females were higher than in NZB males.

DIRECT COOMBS' TEST

At the age of 9 months, NZB males showed a positive direct Coomb's test in 73 per cent, and NZB females in 57 per cent. No positive direct Coombs' test was found in NZB males and females aged 5 months.

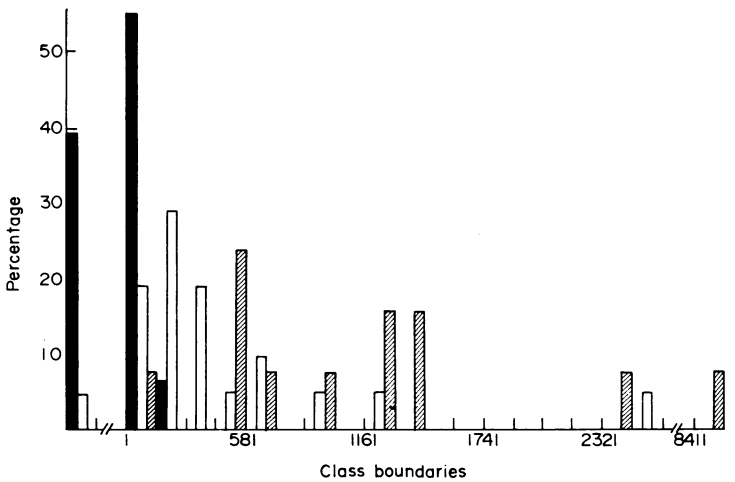

FIG. 4. Frequency distribution of background PFC per spleen in (□) NZB males, (▨) females and (■) controls, aged 9 months.

DISCUSSION

Nine-month-old NZB mice exhibited a prolonged spleen PFC response towards SRBC in comparison to other mouse strains. This phenomenon was found in both sexes; the IgM and the IgG PFC were increased in the primary as well as in the secondary response.

The following mechanism could be involved in the enhanced immune response to SRBC in NZB mice. As suggested by Morton and Siegel (1969), NZB mice could have a higher number of antigen-sensitive cells. If a higher number of precursor cells were present, a dose-dependent response and a higher peak value in the PFC curve would be expected. We did not observe a convincing dose-dependent response, and peak values in NZB mice were not found to be different from those in the other strains. Another explanation for the prolonged immune response could be a disturbance in the feed-back mechanism for the control of antibody synthesis. Henry and Jerne (1968) demonstrated that passively administered specific IgG antibody suppressed the immune response. From the experiments of Walker and Siskind (1968) it is clear that there is a relation between suppression and affinity of the administered antibody. The presence of IgG antibody of a low avidity might account for a prolonged primary and secondary antibody response in NZB mice. Studies along these lines were therefore initiated. These will be presented and discussed in a following paper.

ACKNOWLEDGMENTS

The authors wish to thank Miss J. Slootweg and Mr W. Kamphuis for invaluable technical assistance and Dr O. Brocades Zaalberg for his advice in this study.

REFERENCES

BAUM, J. (1969). 'Increased 7S antibody response to sheep erythrocytes in the 2-month-old NZB mouse.' *Clin. exp. Immunol.*, **5**, 251.

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

BRAVERMAN, J. M. and SLESINSKI, J. (1968). 'Study of autoimmune disease in New Zealand mice.' *J. invest. Derm.* **51**, 274.

BURNET, F. M. and HOLMES, M. C. (1962). 'Immunological function of thymus and bursa of Fabricius.' *Nature (Lond.)*, **194**, 146.

CEROTTINI, J. C., LAMBERT, P. H. and DIXON, F.J. (1969). 'Comparison of the immune responsiveness of NZB and NZB x NZW F1 hybrid mice with that of other strains of mice.' *J. exp. Med.*, **130**, 1093.

CUNNINGHAM, A. J. (1965). 'A method of increased sensitivity for detecting single antibody-forming cells.' *Nature (Lond.)*, **207**, 1106.

CUNNINGHAM, A. J. and SZENBERG, A. (1968). 'Further improvements in the plaque technique for detecting single antibody-forming cells.' *Immunology*, **14**, 599.

DIENER, E. (1966). 'The immune response in NZB and NZB x C3H F1 hybrid mice as measured by the haemolysin plaque technique.' *Int. Arch. Allergy*, **30**, 120.

DRESSER, D. W. and WORTIS, H. H. (1967) 'Localized haemolysis in gel.' *Handbook of Experimental Immunology* (Ed. by D. M. Weir), p. 1054. Blackwell Scientific Publications, Oxford.

EVANS, M. M., WILLIAMSON, W. G. and IRVINE, W. (1968). 'The appearance of the immunological competence at an early age in New Zealand Black mice.' *Clin. exp. Immunol.*, **3**, 375.

HENRY, C. and JERNE, N. K. (1968). 'Competition of 19S and 7S antigen receptors in the regulation of the primary immune response.' *J. exp. Med.*, **128**, 133.

HOLBOROW, E. J. and DENMAN, A. M. (1967). 'Autoimmune disease in inbred mice.' *Clin. exp. Immunol.*, **2**, 761.

HIJMANS, W., RADEMA, H., ES, L. VAN, FELTKAMP, T. E. W., LOGHEM, J. J. VAN, and SCHAAP, P. L. (1969). 'Cryoglobulins in New Zealand black mice' *Clin. exp. Immunol.*, **2**, 227.

JERNE, N. K., NORDIN, A. A. and HENRY, C. (1963). 'The agar plaque technique for recognizing antibody-producing cells.' *Cell-bound Antibodies*. (Ed. by B. Amos and H. Koprowski), p. 109. The Wistar Institute Press, Philadelphia.

MELLORS, R. C., SHIRAI, T., AOKI, T., HUEBNER, R. J. and KRAWCZYNSKI, K. (1971). 'Wild-type Gross leukemia virus and the pathogenesis of the glomerulonephritis of New Zealand mice.' *J. exp. Med.*, **133**, 113.

MONTGOMERY SMITH, J. (1956). 'Studies of chemotaxis in a simple chamber including observations using cells from allergic human subjects and their allergens.' *J. Immunol.*, **77**, 133.

MORTON, J. I. and SIEGEL, B. V. (1969). 'Response of NZB mice to foreign antigen and development of autoimmune disease.' *J. reticuloendothel. Soc.*, **6**, 78.

MUNOZ, J. (1957). 'Production in mice of large volumes of ascites fluid containing antibodies.' *Proc. Soc. exp. Biol. (N.Y.)*, **95**, 757.

PLAYFAIR, J. H. L. (1968). 'Strain differences in the immune response of mice. I. The neonatal response to sheep red cells.' *Immunology*, **15**, 34.

STAPLES, P. J., STEINBERG, A. D. and TALAL, N. (1970). 'Induction of immunologic tolerance in older New Zealand mice repopulated with young spleen, bone marrow or thymus.' *J. exp. Med.*, **131**, 1223.

TALAL, N., STEINBERG, A. D., JACOBS, M. E., CHUSED, T. M. and GAZDAR, A. F. (1971). 'Immune cell cooperation, viruses and antibodies to nucleic acids in New Zealand mice.' *J. exp. Med.*, **134**, 52.

VRIES, M. J. DE, and HIJMANS, W. (1967). 'Pathological changes of thymic epithelial cells and autoimmune disease in NZB, NZW and (NZB×NZW) F_1 mice.' *Immunology*, **12**, 179.

WALKER, J. G. and SISKIND, G. W. (1968). 'Studies on the control of antibody synthesis. Effect of antibody affinity upon its ability to suppress antibody formation.' *Immunology*, **14**, 21.