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Age-related Decline in the Antibody Response to *E. coli* Lipopolysaccharide in New Zealand Black Mice

MARIE-JOSÉ BLANKWATER, LOUISE A. LEVERT AND W. HIJMANS

Institute for Experimental Gerontology TNO, 151 Lange Kleiweg, Rijswijk (ZH), The Netherlands

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Summary. A thymus-independent immune function in ageing NZB and BALB/c mice was compared by measuring the antibody response to *E. coli* lipopolysaccharide (LPS).

Since it was found that 10–13-month-old NZB mice were particularly sensitive to LPS, this antigen had to be detoxified by alkali treatment. The anti-LPS splenic plaque-forming cell (PFC) response of NZB mice decreased with age and was lower than that of BALB/c mice in all age groups studied. The response of 4- and 10-month-old NZB mice showed an irregular time course and a number of mice showed no response. The present results indicate that, besides an impairment of T-cell functions, an impairment on the B-cell level must also be considered in ageing NZB mice.

INTRODUCTION

New Zealand Black (NZB) mice spontaneously develop several abnormal immune reactions. The main abnormalities consist of a Coombs' positive autoimmune haemolytic anaemia, immune complex glomerulonephritis, and antinuclear antibodies (Norins and Holmes, 1964; Mellors, 1966; Howie and Helyer, 1968).

Humoral immune responses to a variety of antigens as well as cell-mediated immune responses have been extensively studied in these mice. Compared to the antibody production in several other strains of mice, NZB mice exhibit hyperresponsiveness as well as hyporesponsiveness, depending on the antigen used (Playfair, 1968; Baum, 1969; Morton and Siegel, 1969; Cerottini, Lambert and Dixon, 1969). Another abnormality is the relative resistance to tolerance induction (Weir, McBride and Naysmith, 1968; Staples and Talal, 1969; Playfair, 1971; Jacobs, Gordon and Talal, 1971).

Cell-mediated immune functions are impaired in ageing NZB mice (Stutman, Yunis and Good, 1968; Rodey, Good and Yunis, 1971). This may be attributed to a decrease in thymus-derived lymphocytes (T cells) (Stutman, 1972) which may be associated with morphological abnormalities of the thymus (de Vries and Hijmans, 1966, 1967; Holmes and Burnet, 1966) or it may be due to antibodies cytotoxic for T cells (Shirai and Mellors, 1971).

Correspondence: Dr M.-J. Blankwater, Institute for Experimental Gerontology TNO, 151 Lange Kleiweg, Rijswijk (ZH), The Netherlands.

Recent studies have given additional information about the antibody response to exogenous antigens in NZB mice. Long-term observations of the immune response to sheep red blood cells (SRBC) have revealed that 9-month-old NZB mice exhibit a prolonged primary and secondary antibody response in comparison to other mouse strains (Elkerbout and Hijmans, 1974a). Low avidity antibodies may account for this prolonged response to SRBC (Elkerbout and Hijmans, 1974b). Using human transferrin as an antigen, Petty and Steward (1972) also observed a relatively lower affinity of antibodies produced in NZB mice as compared to other strains tested. Since both SRBC and transferrin are thymus-dependent antigens, the observed effects can be related to a thymus function.

The purposes of the present study was to investigate the contribution of the thymus-independent immune system (B cells) to the observed deviations from the normal immune reactions in NZB mice. Therefore, age-related changes in the humoral immune response of NZB mice to the thymus-independent antigen *E. coli* lipopolysaccharide (LPS) have been measured and compared with the response to this antigen in BALB/c mice.

MATERIALS AND METHODS

Mice

NZB mice were originally obtained from the Laboratory Animals Centre, Carshalton, Surrey, after the fifty-seventh inbred generation. Following eleven generations at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, they were maintained in our laboratory animal quarters by brother–sister mating. Inbred BALB/c mice obtained from our breeding colony were used for comparison. A shortage of old BALB/c mice necessitated the use of retired breeders.

Nude mice were used to test the thymus-independency of the LPS. Our colony of nude mice was derived from one heterozygous male sent to us by Dr E. M. Pantelouris in 1970. This male was mated with CBA females. The progeny of these matings have been maintained in a closed, but not inbred, group under conventional conditions.

The animals were fed standard AM mouse pellets (Hope Farms, Holland) with free access to tap water.

Approximately equal number of male and female mice were used in the experimental groups.

Antigen and immunization

Lipopolysaccharide (LPS) of *E. coli* 055:B5 prepared according to the phenol-extraction method (Westphal, Luderitz and Bister, 1952) was obtained from Difco Laboratories, Detroit, Michigan, U.S.A. Detoxification was performed with weak alkali as described by Britton (1969); these preparations were dissolved in saline and were injected into a tail vein.

Antigen coating of SRBC

Sheep red blood cells (SRBC) were obtained from our animal colony and stored in sterile Alsever's solution. The method of Andersson and Blomgren (1971) was used for coating the SRBC with LPS. Briefly, LPS dissolved in phosphate-buffered saline (1 mg/ml) was boiled for 2 hours while the pH was adjusted to pH 8 by adding 0·1 M NaOH. To 1·5 ml of boiled LPS solution, 0·5 ml of packed SRBC was added. This mixture was incubated at 37° for 45 minutes. A new batch of coated SRBC was prepared for each test.

The extent of coupling was routinely tested by measuring the haemagglutination titre of a standard mouse anti-LPS serum with each batch of SRBC–LPS.

Plague-forming cell (PFC) assay

Antibody-forming spleen cells were detected by an agar-free plaque technique (Cunningham and Szenberg, 1968) with some modifications as described by Zaalberg, van der Meul and Twisk (1968). Guinea-pig serum was used as the source of complement. Naturally occurring antibodies to SRBC were removed by absorption with SRBC. Absorption of the guinea-pig serum with agarose according to the method of Cohen and Schlesinger (1970) appeared to be effective for removing the antibodies against LPS (Zaalberg, personal communication).

Each spleen cell suspension was assayed on both control and coated SRBC and the number of anti-SRBC–PFC were subtracted to give the number of anti-LPS–PFC.

Haemagglutination assay

Serum samples were serially diluted two-fold in 0·025 ml of PBS. Dilutions were performed by an Automatic Diluter (Cooke Engineering Company, Alexandria, Virginia, U.S.A.) in U-shaped microtitre plates. An equal volume (0·025 ml) of a 2 per cent SRBC suspension in PBS was added. The plates were incubated at 37° for 1 hour and were stored at 4° overnight. Individual cells were examined under a microscope with a magnification of ×25.

Results are expressed as $1/\log_2$ of the highest dilution of serum giving positive agglutination.

Calculations and statistical analysis

The number of antibody-forming cells was logarithmically transformed and geometric means and 95 per cent confidence limits calculated; *P* values were determined by Student's *t*-test. In the comparison of the means of any two groups of observations, a level of significance of 0·05 was chosen.

RESULTS

SENSITIVITY OF NZB MICE TO LPS

Although the dose of LPS which is sufficient for induction of an immune response is not toxic for other strains of mice, it appeared during the first experiments that even small quantities (1 and 5 μg of LPS) were highly toxic to 10–13-month-old NZB mice (Table 1).

TABLE 1

SENSITIVITY OF NZB AND BALB/C MICE TO THE LETHAL EFFECT OF LPS

Mouse strain	Age (months)	Number of animals	LPS (μg)	Percentage of dead animals
NZB	4	40	10	0
BALB/c	4	50		0
NZB	10	35	5	43
BALB/c	10	25		4
NZB	13	125	1	49
BALB/c	13	90		0

The mice died within 24–48 hours after administration of the LPS. Young NZB and BALB/c mice were not sensitive to the lethal effect of LPS. Therefore, it was necessary to decrease the toxicity in order to be able to study the immune response of NZB mice to LPS without selecting animals that were not susceptible to the substance. Detoxification was performed by treating the LPS with weak alkali (Britton, 1969). This preparation was no longer toxic to NZB mice.

THYMUS-INDEPENDENCY OF LPS AND DETOXIFIED LPS

To establish the thymus-independency of detoxified LPS, the antibody response of athymic nude (nu/nu) mice and their normal litter mates (+/?) to LPS and detoxified LPS was compared. This was done on the peak day (day 4) of the PFC response to LPS in nu/nu and +/? mice. The number of PFC in these two groups did not differ significantly (Table 2). This indicates that neither the response to LPS nor the response to detoxified

TABLE 2

ANTI-LPS RESPONSE IN NUDE MICE AND NORMAL LITTERMATES

Experiment number*	Antigen	Dose (μg)	Number of animals		PFC per spleen ($\times 10^{-3}$) 4 days after immunization	
			nu/nu†	+/?‡	nu/nu	+/?
1	LPS	5	4	4	102 (86–122)	120 (106–135)
2	Detoxified LPS	5	5	5	30 (24–37)	24 (19–31)

Geometric mean, upper and lower limits of S.E.

* P values between nude and normal litter mates: experiment 1 and experiment 2 $P > 0{\cdot}05$.

† nu/nu = Nude mice.

‡ +/? = Phenotypically normal litter mates of nude mice.

LPS requires the participation of thymus-derived cells. Untreated LPS gave a higher response than did detoxified LPS.

SPLENIC ANTI-LPS RESPONSE

The splenic PFC response to LPS was measured in 2-, 4- and 10-month-old NZB and BALB/c mice. The mean survival time of NZB mice is about 14 months (Howie and Helyer, 1968) so that a 10-month-old NZB mouse can be considered old, in contrast to a 10-month-old BALB/c mouse, which is middle-aged. Consequently, the LPS response was also determined in 19–21-month-old BALB/c mice. On the basis of a limited number of pilot experiments, a dose of 5 μg of detoxified LPS was chosen. The time courses of the LPS response of NZB and BALB/c mice are given in Fig. 1. Following immunization, the BALB/c mice showed an exponential increase in antibody forming cells with a peak response on day 4. The peak response to LPS in BALB/c mice increased with age. A significant difference was found between 2- and 4-month-old and 4- and 10-month-old mice, but not between 10- and 19–21-month-old BALB/c mice.

Although the number of antibody-forming cells from 2-month-old NZB mice was lower, the same time course with a peak on day 4 as compared to the BALB/c mice was observed.

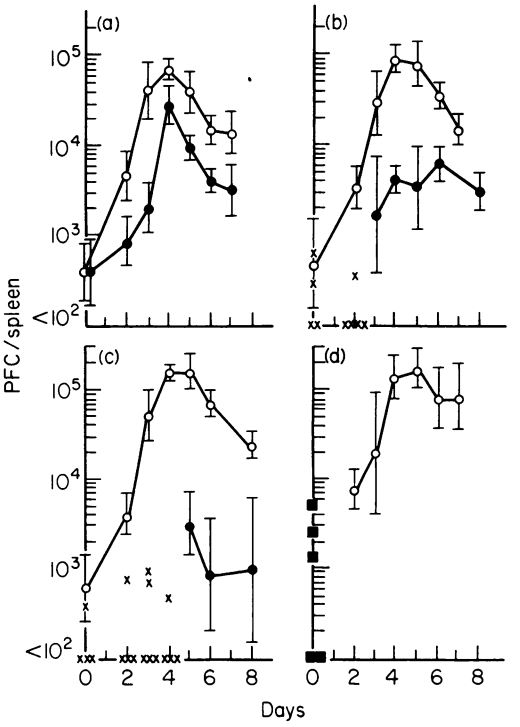

FIG. 1. Development of plaque-forming cells in NZB (●, mean and ×, individual values) and BALB/c (○, mean and ■, individual values) mice at various days after intravenous injection of 5 μg of detoxified LPS. Each circle represents the geometric mean of four to five mice. The 95 per cent confidence limits are indicated by bars. (a) Two-month-old mice. (b) Four-month-old mice. (c) Ten-month-old mice. (d) Nineteen to 21-month-old mice.

Furthermore, the number of background PFC to LPS (day 0) was the same in 2-month-old BALB/c and NZB mice. Four- and 10-month-old NZB mice gave the poorest response of the different groups studied. They did not show a clear time course. Mice that developed fewer than 100 PFC per spleen were considered as non-responder mice. Non-responder mice were observed at 2 and 3 days after immunization in 4-month-old NZB mice and at 2, 3 and 4 days after immunization in 10-month-old NZB mice. This reflects a slow start of the immune response in these mice. Furthermore, the variations in the number of antibody-forming cells within these age groups of NZB mice were considerable. No correlation could be observed between the number of PFC and the spleen weights. This also applies to the 10-month-old NZB mice in which spleen weight increased.

SERUM ANTIBODY TITRE AGAINST LPS

Additional information was acquired by assaying the serum of BALB/c mice and NZB mice of different ages for antibody titres against LPS (Fig. 2). The antibody levels of BALB/c mice were always higher than those of NZB mice. The antibody titres of BALB/c mice increased with age, whereas the titres of NZB mice decreased; 19–21-month-old BALB/c mice gave the best response. In general, therefore, the same tendency as seen with PFC response was observed.

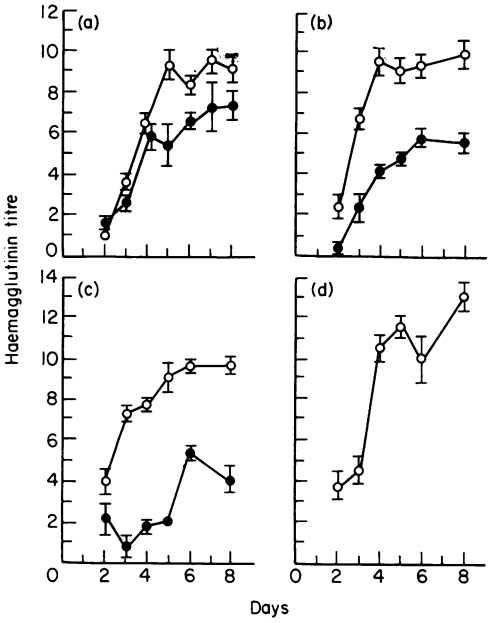

FIG. 2. Development of serum antibody titres in NZB (●) and BALB/c (○) mice at various days after an intravenous injection of 5 μg of detoxified LPS. Each circle represents the mean of four to five mice. The upper and lower limits of S.E. are indicated by bars. (a) Two-month-old mice. (b) Four-month-old mice. (c) Ten-month-old mice. (d) Nineteen to 21-month-old mice.

DISCUSSION

LPS is a component of a bacterial endotoxin which is known to cause a multitude of physiological disturbances. Genetic differences may account for the difference in sensitivity of NZB and BALB/c mice to LPS, since this sensitivity is controlled by complex genetic mechanisms (Robson and Vas, 1972). Chedid (1973) has found that mice became hyper-reactive to endotoxins after either immunosuppression or immunostimulation, indicating that susceptibility to endotoxin may be enhanced by an immunological imbalance. Therefore, the immunological disorder observed in aged NZB mice may also account for the high sensitivity of old NZB mice to LPS. Experiments are in progress to determine whether the lethal effect of LPS could be caused by impurities (nucleic acids or proteins) in the LPS preparation.

NZB mice are no longer susceptible to the toxic effect of LPS after the LPS has been treated with weak alkali (Britton, 1969). This treatment reduces the size of the molecule, presumably by splitting the ester bonds.

The thymus-independence of the immune response to LPS is well established for the mouse (Andersson and Blomgren, 1971; Möller and Michael, 1971). By comparing the immune response to LPS and detoxified LPS in nude mice and their normal litter mates, it appeared that both LPS and detoxified LPS behave as thymus-independent antigens. Therefore, the antibody response to the latter can also be considered as a B-cell function only.

The results presented in this paper demonstrate that, if we compare the humoral immune response of NZB and BALB/c mice to LPS, NZB mice develop a lower response

which further decreases with age as early as the 2nd month. Moreover, the time courses of the response in 4- and 10-month-old NZB mice are irregular and non-responder mice are observed during the first days after immunization. Since antibody formation against LPS is a B-cell response, our results are indicative of a functional loss of cells forming antibodies to LPS in aged NZB mice and may reflect a progressive defect in the bone marrow-derived lymphocyte population.

It has been suggested that the bone marrow-derived lymphocyte population (B cells) is abnormal in NZB mice. Staples, Steinberg and Talal (1970) reported that the B cells of these mice are less easily made tolerant to bovine gamma-globulin than those of normal mice, even in the presence of competent thymus-derived cells. De Jesus, Holborow and Brown (1972) produced evidence that ageing NZB mice show an increasing inability to localize aggregated human gamma-globulin complexes in germinal centres, a function chiefly attributed to B cells.

Furthermore, our results are in accordance with the data of Purves and Playfair (1973) who also found a hyporeactivity of 3–6-month-old NZB mice to two other thymus-independent antigens, pneumococcal polysaccharide type 3 (SIII) and bacterial levan. Barthold, Kysela and Steinberg (1974), however, reported an increase in antibody formation to SIII in ageing NZB mice, which they suggest to be due to a loss of suppressor T-cell function.

The existence of regulatory T cells was first suggested by the enhanced antibody response obtained when anti-lymphocyte serum (ALS) was given with the SIII (Baker, Barth, Stashak and Amsbaugh, 1970). This regulatory effect of T cells is also observed in the response to polyvinylpyrrolidone (PVP), another thymus-independent antigen (Rotter and Trainin, 1974). However, no evidence has been presented so far that suppressor T cells play a regulatory role in the antibody response to LPS. This response is not affected by ALS treatment (Veit and Michael, 1972). It appears, therefore, that LPS, SIII, and PVP are all antigens that do not require thymus-derived helper cells for the induction of the immune response; however, in contrast to SIII and PVP, the magnitude of the antibody response to LPS is not controlled by thymus-derived regulatory cells. All these data, therefore, indicate that the age-related decline in the immune response to LPS in the NZB mouse is a consequence of a decrease in B-cell function only.

There is also a decline of functions attributed to thymus-derived cells in ageing NZB mice, e.g. allograft rejection (Teague, Yunis, Rodey, Fish, Stutman and Good, 1970), response to mitogenic antigens (Leventhal and Talal, 1970; Rodey *et al.*, 1971), and graft-versus-host reactions (Cantor, Asofsky and Talal, 1970). Furthermore, Bach, Dardenne and Salomon (1973) reported the absence of serum 'thymic activity' in adult NZB mice.

In summary, these data support the idea that a combined defect at both the B- and T-cell level is responsible for the immunological disorders observed in ageing NZB mice. This combined defect may be a consequence of a defect in haemopoietic stem cell differentiation in these animals (Warner and Moore, 1971).

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