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The immune response in NZB mice of different ages to thymus-dependent and thymus-independent phosphorylcholine antigens

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Summary. This study was designed to examine aberrations of immune responses in autoimmune NZB strain mice during ageing, at the level of individual B cell clones. The response to phosphorylcholine (PC) was chosen because murine responses to PC are restricted to a few B cell clones and can be characterized with idiotypic markers. Responses to both thymus-dependent (TD) and thymus-independently (TI) PC-containing antigens were measured in mice ranging from 1 to 62 weeks of age. We found that: (1) TD responses to phosphorylcholine keyhole limpet haemocyanin (PC-KLH) decreased markedly (about 17-fold) between 28 and 62 weeks of age. TI responses to the R36a strain pneumococcus decreased only slightly during the same period. (2) The PFC responses to both antigens became markedly prolonged in mice older than 24 weeks. (3) The NZB response to either antigen is essentially monoclonal, as measured by inhibition of PFC with specific anti-idiotype serum and PC hapten. No age-related alteration in avidity or idiotype expression was observed.

Our results demonstrate that no aberrant PC-reactive B cell clones appear in old NZB, and lend support to the notion that the abnormalities observed are due to defective regulatory mechanisms.

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INTRODUCTION

Previous studies of the humoral immune responses of New Zealand black (NZB) mice have utilized a wide array of unrelated antigens to study thymus-dependent (TD) and thymus-independent (TI) responses as a function of age (Barthold, Kysela & Steinberg, 1974; Naor, Bonavida, Robinson, Shibata, Percy, Chia & Barnett, 1976). However, none of these studies attempted to monitor a restricted set of antigen-reactive clones in an age-dependent fashion.

In the mouse, phosphorylcholine (PC) antigens induce IgM antibodies of restricted heterogeneity as defined by their affinity and idiotypic markers (Claflin, 1976). Also, thymus-dependent and thymus-independent PC antigens are available which stimulate functionally different subpopulations of PC-specific B cells (Quintáns & Cosenza, 1976). By following the humoral responses to TD and TI forms of PC-containing antigens in NZB mice, we have been able to monitor the behaviour of a highly restricted and well-defined immune response in a model system for autoimmunity. The appearance of aberrant clones was studied by measuring inhibition of PFC by free PC hapten and by specific anti-idiotype serum.

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MATERIALS AND METHODS

Animals

Female NZB mice of various ages (8–62 weeks) were used throughout these experiments. Mice were obtained from Jackson Laboratories (Bar Harbor, Maine). In addition, newborn mice (aged 1–2 weeks) from our breeding colony were used in some experiments.

Antigens and immunizations

Two PC antigens were used. R36a and PC-KLH. R36a is a TI (Quintáns & Cosenza, 1976) *Streptococcus pneumoniae* vaccine prepared as described previously (Cosenza & Köhler, 1972). PC coupled to keyhole limpet haemocyanin (PC-KLH) is a soluble TD antigen (Quintáns & Cosenza, 1976). PC was coupled to KLH using paradiazonium phenylphosphorylcholine (DPPC) (Chesebro & Metzger, 1972) as reported (Quintáns & Cosenza, 1976). The preparation of PC-KLH used in the present experiments contained 2 PC groups per 10^5 Daltons of KLH.

Mice of 8 weeks or older were immunized intravenously with either 10^9 formalinized R36a bacteria or 200 μg PC-KLH. Mice aged 1–2 weeks were injected intraperitoneally with either 5×10^8 formalinized R36a bacteria or 50 μg PC-KLH.

Coating of SRC and PFC technique

PC-coated SRC were prepared as described (Claflin, Lieberman & Davie, 1974) and used as target cells in the Cunningham-Szenberg (Cunningham & Szenberg, 1968) modification of the haemolytic plaque technique (Jerne & Nordin, 1963). The results shown represent the means of PFC assays of individual spleens plaqued in quadruplicate and expressed as PFC/10^6 spleen cells as well as PFC/spleen.

Plaque inhibitions

Avidity of antibody secreted was measured by inhibiting PFC with free hapten. Phosphorylcholine chloride (Sigma, St Louis, Missouri) was incorporated into the plaquing mixture at various molar concentrations and the number of PFC in triplicate chambers was averaged and expressed as percentage PFC inhibited.

Inhibitions of PFC with anti-idiotypic serum were carried out in a similar manner with a heterologous serum described below.

Anti-idiotype serum

Strain 13 guinea-pigs (Bionetics, Frederick, Maryland) were injected in the hind foot pads with 100–400 μg of TEPC-15 myeloma protein, purified according to Chesebro & Metzger (1972) in Freund's complete adjuvant and were exsanguinated 4 weeks later. Antiserum obtained in this fashion routinely cross-reacted with other PC binding myeloma proteins representing other idiotypes (MOPC-167 and McPC-603). After passage of anti-TEPC-15 serum through columns containing normal mouse immunoglobulin (previously adsorbed on PC-Sepharose 4-B (Chesebro & Metzger, 1972) and MOPC-167 myeloma protein covalently linked to Sepharose 4-B

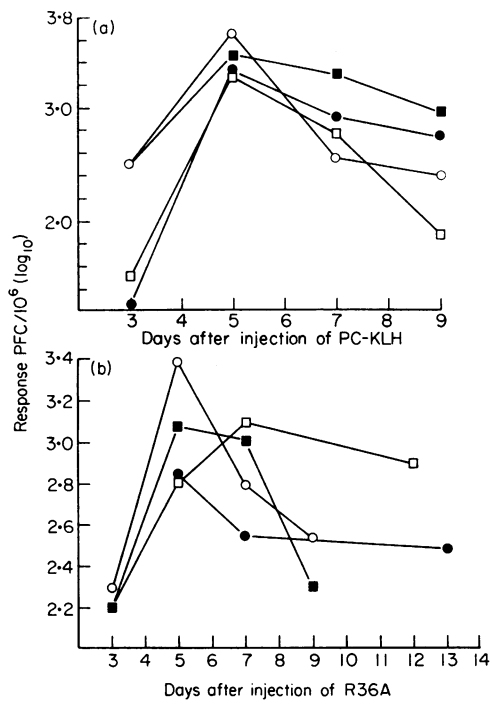

Figure 1. Kinetics of PFC responses to PC antigens in NZB mice of different ages. Groups of NZB mice ranging from 8 to 53 weeks of age received intravenous injections of either PC-KLH or R36a vaccine. PFC responses to PC were measured on several different days after antigen injection. Injections were staggered so that all responses in a given age group were measured on the same day. Each point represents the mean PFC in a pool of spleen cells from 2 to 4 mice. The standard error of the PFC counts was always less than 10% of the mean. (a) Responses to PC-KLH in mice aged 8 weeks (□), 9 weeks (○), 24 weeks (●), and 53 weeks (■). (b) Responses to R36a vaccine in mice aged 8 weeks (○), 9 weeks (■), 25 weeks (●), and 49 weeks (□).

(Cuatrecasas, 1970), no reactivity against MOPC-167 or McPC-603 was observed in passive haemagglutination and haemagglutination inhibition assays (Lieberman & Humphrey, 1971). Adsorbed guinea-pig anti-TEPC-15 serum inhibited anti-PC plaques but not anti-SRC plaques.

RESULTS

Kinetics of PFC responses to PC antigens in NZB mice of different ages

Groups of NZB mice ranging from 8 to 53 weeks of age received intravenous injections of either PC-KLH (TD) or R36a vaccine (TI) (Quintáns & Cosenza, 1976). PFC responses to PC were measured on days 3, 5, 7 and 9 post-injection. Additionally, older animals (24 weeks or older) injected with R36a were assayed on days 12 and 13 post-injection. The injections were done on different days so that all of the responses in a given group were measured on the same day.

With only one exception, the PFC response to PC-KLH (Fig. 1a) and to R36a (Fig. 1b) were both maximal at day 5 post-injection. Mice approximately 50 weeks of age or older immunized with R36a displayed a delayed peak response; this delay shifted the peak from day 5 to day 7. In addition, the number of PFC measured upon challenge with PC-KLH or R36a in mice 24 weeks or older did not decrease at the same rate as those of 8–9 week-old mice; i.e. responses of older mice were sustained. For instance, young mice injected with PC-KLH showed a 25-fold drop from day 5 to day 9 post-injection, while in older mice only a 3-fold drop was observed over the same period of time. Likewise, the response to R36a in young mice decreased 7-fold from day 5 to day 9, whereas the number of PFC in old mice decreased only 1·5-fold even when PFC were measured as late as day 13.

Responses to PC-KLH and R36a as a function of age

Once it was determined that peak anti-PC responses occur on day 5 after antigen challenge (with the exception noted above) we proceeded to evaluate the capacity of NZB mice of different ages to respond to PC-KLH and R36a. The results are presented in Table 1 and establish the following points: (1) PC-KLH is more immunogenic than R36a at all ages tested. (2) Maximum responses to both antigens occur between 8 and 28 weeks of age. (3) After 28 weeks of age there is a rapid decline in the response to TD antigen (PC-KLH) and a marked increase in the scatter of the responses of individual mice. The responses of a total of 22 mice 8–28 weeks

Table 1. Anti-PC responses in NZB mice of different ages

Age (weeks)	Antigen	No. of animals	Log_{10} PFC/spleen	% Maximal response	Log_{10} PFC/10^6	% Maximal response
1	PC-KLH	10	3·42 ± 0·51	1	2·07 ± 0·55	5
2	PC-KLH	5	4·15 ± 0·40	4	2·42 ± 0·36	12
8–12	PC-KLH	12	5·59 ± 0·28	100	3·33 ± 0·34	98
22–28	PC-KLH	8	5·37 ± 0·14	72	3·34 ± 0·30	100
40–48	PC-KLH	12	4·70 ± 0·64	15	2·41 ± 0·76	12
54–62	PC-KLH	7	4·30 ± 0·45	6	2·25 ± 0·74	8
1	R36a	7	2·65 ± 0·29	1	1·39 ± 0·32	7
2	R36a	8	3·13 ± 0·46	2	1·61 ± 0·53	12
8–12	R36a	15	4·72 ± 0·27	95	2·53 ± 0·30	95
22–28	R36a	10	4·74 ± 0·46	100	2·54 ± 0·46	100
40–48	R36a	10	4·69 ± 0·41	89	2·39 ± 0·35	71
54–62	R36a	4	4·24 ± 0·34	32	2·29 ± 0·37	56
54–62	R36a	4	4·45 ± 0·61*	51	2·28 ± 0·68	55

NZB mice of various age groups were injected with PC-KLH or R36a i.v. and PFC responses measured 5 days later unless indicated otherwise in the table. Results are expressed as the geometric means of log transformed data ± s.e. Student's *t*-test for the means of age groups 8–12 and 54–62 weeks gave $P < 0·001$ for PC-KLH. The same age groups immunized with R36a gave $P > 0·2$.

* Day 7 responses.

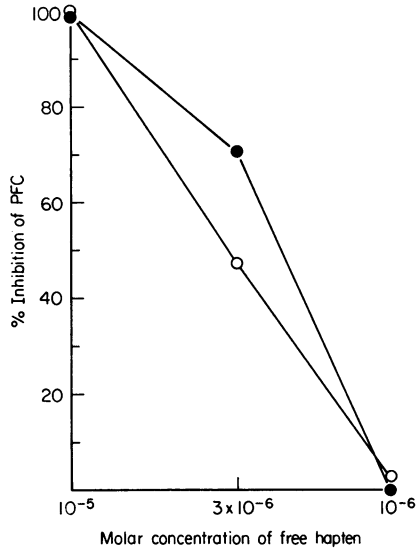

Figure 2. Inhibition of anti-PC plaque responses of young and old NZB mice by free hapten. Young (18 weeks) and old (62 weeks) NZB mice were injected with PC-KLH and 5 days later PFC assays were performed in the presence of various concentrations of phosphorylcholine chloride. The results are expressed as per cent inhibition of PFC as a function of hapten concentration for mice aged 18 weeks (●) or 62 weeks (○).

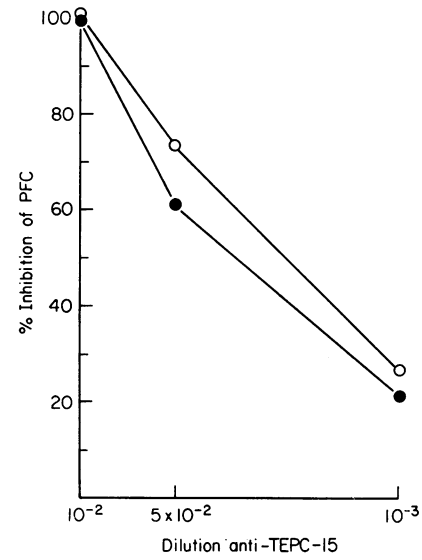

Figure 3. Inhibition of anti-PC plaque responses of young and old NZB mice by anti-idiotype. Young (18 week) and old (62 week) NZB mice were injected with PC-KLH, and 5 days later PFC assays were performed in the presence of various dilutions of anti-TEPC-15 serum. The results are expressed as per cent inhibition of PFC as a function of antiserum dilution for mice aged 18 weeks (●) or 62 weeks (○).

of age ranged between 700 and 7000 PFC/10^6 (10-fold spread), whereas those of 19 mice older than 40 weeks ranged between 5 and 2400 PFC/10^6 (500-fold spread). (4) The TI anti-PC responses induced by R36a remain stable up to 40–48 weeks of age and then start to decline. Moreover, no age-related increase in variation between individual mice was noted; young mice (8–28 weeks) ranged from 27 to 1031 PFC/10^6 spleen cells while old mice (40–62 weeks) ranged from 40 to 1360 PFC/10^6 spleen cells (a 35-fold spread in both cases).

Plaque inhibition with PC hapten and with anti-idiotype

Fig. 2 shows the results of a representative experiment measuring inhibition with various concentrations of PC hapten of PFC obtained from young or old mice injected 5 days previously with PC-KLH. The two inhibition curves were essentially identical. Several other experiments using mice injected with PC-KLH or R36a confirmed that the inhibition curves for young and old NZB mice were not significantly different. In addition, comparable inhibition curves were obtained with PFC obtained from BALB/c mice when the same indicator cells were used; i.e. 100% PFC inhibition at 10^{-5} molar PC and 0% inhibition at 10^{-6} molar PC. These results agree with our previous observation that TD and TI anti-PC responses display identical avidity (Quintáns & Cosenza, 1976).

We also performed experiments to determine whether idiotype expression was altered as a result of age. To this end, guinea-pig anti-TEPC-15 serum was used to inhibit anti-PC PFC in spleen cells of young and old NZB mice injected with PC-KLH. It may be seen in Fig. 3 that mono-specific anti-idiotypic serum to the TEPC-15 idiotype caused 100% inhibition of PFC in both age groups. Several other experiments confirmed that the predominant idiotype of anti-PC PFC in young and old NZB was the TEPC-15 idiotype (range 70–100% inhibition of PFC with anti-TEPC-15 serum).

DISCUSSION

The response of NZB mice to various antigenic challenges has been studied extensively. An early maturation of their capacity to respond to SRC (Playfair, 1968), hyper-responsiveness to SRC as young adults (Baum, 1969), hypo-responsiveness in old animals (Barthold, Kysela & Steinberg, 1974) and difficulty in inducing tolerance (Weir, McBride & Naysmith, 1968; Staples & Talal, 1969; Staples, Steinberg & Talal, 1970; Playfair, 1971) have all been reported. However, Cerottini *et al.* (1969) in comparing NZB with three other strains in their responses to several antigens found that hyper- and hypo-responsiveness in NZB were relative. Other studies have shown that age-dependent changes in NZB mice affect TD and TI responses differently; for example, the anti-SRC response, which is largely TD, is markedly reduced in old mice, whereas that to SIII, a TI antigen under T-suppressor cell regulation (Baker, Barth, Stashak & Amsbaugh, 1970) is enhanced (Barthold, Kysela & Steinberg, 1974). These results and others (Talal & Steinberg, 1974) support the notion of a loss of helper and suppressor T cells with age.

In the present study we have examined the capacity of NZB mice (ranging from 1 to 62 weeks of age) to mount humoral immune responses to PC antigens. We have chosen this system because murine anti-PC responses are known to display restricted heterogeneity in normal mouse strains (Claflin, 1976), because idiotype markers are available to monitor responses at the clonal level (Cosenza & Köhler, 1972), and because TD and TI forms of PC-antigens are available.

We first measured the kinetics of the anti-PC PFC response as a function of time after antigen presentation in NZB mice of various ages. In general, the peak PFC response occurred on day 5, regardless of age. However, we noted a marked age-dependent prolongation of the PFC response to both the TD and TI antigens. This finding extends previous observations (Elkerbout & Hijmans, 1974) that NZB mice display sustained responses to SRC, while several other strains do not.

We have found that the capacity to respond to PC-KLH and R36a is manifest in NZB mice 5 days after birth, at approximately the same age as in BALB/c mice (Jose Quintáns & John McKearn, unpublished). NZB responses to both antigens increase similarly until 10–24 weeks of age and thereafter follow somewhat independent fates; the response to PC-KLH displays a marked, steady decline with age, whereas that to R36a remains unaltered until 40–48 weeks of age. Furthermore, the range of responses in individual old mice to PC-KLH, but not to R36a, is considerably greater than that of young NZB.

Our conclusions in the PC antigenic system are in general agreement with those of Naor *et al.* (1976) using TNP antigens. They observed a greater decline in the magnitude of responses to TNP-SRC (TD) than to TNP-MRC (mouse red cells), which is a TI antigen, although they failed to exclude that this decline was due to a shift in peak responses. At variance with our results is their observation of the decrease in avidity of TD responses in ageing mice, which is attributed to T helper cell malfunction and selective stimulation of low affinity clones under conditions of defective T cell help. In our case, antigens which induce monoclonal responses were used, so that defects in helper or other auxiliary cell types would lead to a decrease in magnitude of the response, but not to a change in avidity. It should also be noted that, whereas responses to SRC have virtually disappeared in NZB at 50 weeks of age or older, we can still detect a significant TD response to PC-KLH at that age. We attribute this to the nature of the carrier, since KLH is known to be a potent stimulator of T cells and would tend to minimize deficiencies in the T helper cell compartment (Waldman, Lefkovits & Quintáns, 1975).

There is general agreement in published reports on the decline with age of responses to TD antigens in NZB (Barthold, Kysela & Steinberg, 1974; Naor *et al.*, 1976), although there are some discrepancies with regard to TI responses. Thus, it was shown that the TI responses to LPS (Blankwater, Levert & Hijmans, 1975) and TNP on mouse red cells (Naor *et al.*, 1976) and to R36a (this paper) are depressed in old NZB, whereas the response to SIII increases with age, a change attributed to loss of suppressor cells (Barthold, Kysela & Steinberg, 1974). Our TI antigen, R36a, is not known to be under the regulation of suppressor T cells (Hopkins, 1975), and therefore one would not expect that changes in T cell suppressor activity would affect R36a-induced responses. In fact, we have shown that for the age period covered in our studies (62 weeks) the TI anti-PC responses remain relatively stable.

Our results demonstrate that NZB mice of different ages respond to PC-KLH (TD) and to R36a

(TI) with anti-PC responses of restricted heterogeneity, as shown by PFC inhibition with free hapten and with anti-idiotypic serum. These studies showed that the NZB anti-PC responses are remarkably similar to BALB/c anti-PC responses in avidity and idiotype. In agreement with Sher & Cohn (1972), we have found that the TEPC-15 marker (Potter & Lieberman, 1970) is the predominant idiotype in NZB anti-PC antibodies. However, we were unable to detect changes in antibody avidity or idiotype in anti-PC responses of old NZB, i.e. no aberrant B cell clones appear in old mice. The results of PFC inhibitions with anti-idiotypic serum demonstrate clearly that there is no emergence of new clones in old NZB. It is not excluded (and it is likely) that a loss of PC-specific B cell clones occurs with age. However, formal proof for this would require an analysis at the precursor cell level under limiting dilution conditions for B cells from old NZB in the presence of excess T cell help and normal macrophages. It should be noted that DeHeer & Edgington (1975), in their studies of the response to SRC, concluded that old NZB had an intrinsic defect of B cells. Since their conclusions were based on differences in avidity in PFC of NZB and C3H mice, these differences can be accounted for by defects at the T cell level (Davie & Paul, 1973). Their results as well as ours suggest altered regulation of B cell function with age rather than changes in the B cells themselves.

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