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Age-dependent Changes in the Relative Affinity of Anti-Dinitrophenyl Antibodies in Mice

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Summary. The affinity of anti-DNP antibodies produced by mice of various ages has been studied at the cellular level by the plaque inhibition technique. The affinity of PFC produced shortly after a single injection of antigen was found to increase during the first 6 weeks of life. The responses of 1- and 2-week-old animals also showed apparently restricted heterogeneity.

The difference in the affinity of anti-DNP–PFC between young and adult mice could not be attributed to different (serum) levels of antigen or to differences in the rate of maturation of affinity during the immune response.

Cell transfer experiments suggested that the age-dependent increase was due to a change in the population of antibody-forming (B) cell precursors and not to a progressive improvement of T-cell function. This finding is interpreted as favouring somatic mutation theories of antibody diversity.

INTRODUCTION

The determination of the relative affinity of antibodies at the cellular level by means of inhibition of plaque-forming cells (PFC) has become a widely used technique in the past few years. Using this method the relative affinity (avidity for multideterminant antigens) of antibodies to DNP (Yamada, Yamada and Hollander, 1970; Davie and Paul, 1972; Miller and Segre, 1972; Huchet and Feldman, 1973), arsanilic acid (Wu and Cinader, 1972), type III pneumococcal polysaccharide (Baker, Prescott, Stashak and Amsbaugh, 1971), bovine serum albumin (Andersson, 1970) and the a1 allotype of rabbit immunoglobulin (Segre, Segre and Inman, 1969) have been determined. The average relative affinities derived from the plaque inhibition method and the modified Farr test on serum antibodies have been compared and show a linear correlation (Miller and Segre, 1972).

In this paper the plaque inhibition technique has been used to follow the relative affinity of anti-DNP antibodies produced during the primary immune response in (C57Bl × BALB/c)F1 mice of various ages. A marked increase of affinity was found to occur during early life, and shown to reflect changes in the populations of anti-DNP precursors.

MATERIALS AND METHODS

Mice

C57Bl, BALB/c and (C57Bl × BALB/c)F1 mice were bred in our laboratory from inbred stocks derived from the Laboratory Animals Centre, Carshalton.

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Antigen

Dinitrophenylated keyhole limpet haemocyanin (DNP–KLH) was prepared by incubating 200 mg of KLH (Calbiochem, Ltd) with 0·4 ml of fluoro-dinitrobenzene (FDNB) (B.D.H., Ltd) in phosphate-buffered saline (PBS) for 20 minutes at 37°. Unreacted FDNB was removed by chromatography on Sephadex G-25 and extensive dialysis against PBS.

The substitution ratio calculated from optical density readings at 280 nm and 360 nm was 250 moles of DNP per mole of KLH (assumed molecular weight 4×10^6).

Antigen was freshly alum precipitated for immunization and given with 2×10^9 *B. pertussis* organisms.

Preparation of cells

Spleen cells. Mice were killed 7 days after immunization, and their spleens removed and teased into cold Eagle's medium buffered by the addition of 15 mM Hepes. After mixing with a Pasteur pipette the suspension was allowed to stand for 10 minutes for clumps to settle and the remaining cells washed twice before use.

Thymus cells. Thymuses from mice aged 6–12 weeks were used. Cells were prepared as above.

'B' spleen cells. (C57Bl × BALB/c)F1 mice thymectomized at 4 weeks of age were irradiated (850 rad) 2 weeks later and restored with an intravenous injection of 10^6 sygeneic bone marrow cells. Their spleens, removed 12 weeks after repopulation, were used as a source of 'B' spleen cells.

PFC assay

Anti-DNP–PFC were assayed by the technique of Cunningham and Szenberg (1968). SRBC were coated with DNP by incubating with a suitable amount of either DNP-labelled chicken anti-SRBC gamma-globulin (DNP_6–CGG) as described by Silver, Miller and Warner (1971), or the dinitrophenylated Fab fragment of rabbit anti-SRBC IgG (DNP_5–Fab). The DNP chicken anti-SRBC gamma-globulin was used in all experiments except where specifically stated in the tables. Indirect PFC were developed by the addition of a rabbit anti-mouse immunoglobulin serum. At the dilution employed this serum inhibited 95 per cent of direct (2-day) PFC.

PFC obtained with uncoated SRBC were subtracted from those with DNP–SRBC to give the number of specific anti-DNP–PFC.

Plaque inhibition assay

Anti-DNP–PFC were inhibited by the inclusion of DNP–ε-lysine (B.D.H., Ltd) into the chambers; the extent of inhibition depended on the concentration of DNP–ε-lysine added. PFC against SRBC were not inhibited at DNP–ε-lysine concentrations of up to 10^{-3} M.

The concentration of DNP–ε-lysine producing 50 per cent inhibition of anti-DNP (I_{50}) was taken as a measure of the relative affinity of the PFC. Theoretical analysis by DeLisi and Goldstein (1974) indicates that this method should yield an affinity distribution which corresponds well with the actual distribution of affinities. The conditions under which this is true are: (1) that univalent attachment between type-2 (both sites free) antibody and RBC epitope is quickly followed by the establishment of a second bond; (2) that such bivalent binding is irreversible during times of experimental interest, with the

provision that secretion rates are symmetrically distributed among the affinity groups. To fulfil the first condition clearly requires a relatively high density of red cell-bound hapten, in agreement with the work of Pasanen (1971) who found that whilst heavily substituted RBC detected PFC of both low and high affinity, lightly substituted RBC detected only high affinity PFC.

Although in our experiments we have not established the actual extent of substitution of the RBC we are confident that it is sufficiently high to comply with condition (1) above. This is because no increase in the amount of DNP–CGG or DNP–Fab used for conjugation over the amount routinely used has allowed the detection of extra PFC.

To ensure that conjugation was as standard as possible we used single batches of DNP–CGG and DNP–Fab, and a set amount of one of these was always used to sensitize the same volume of RBC. The extent of conjugation was checked by passive haemagglutination using a goat anti-DNP serum, batches of DNP-coated RBC produced on different days always agglutinated to the same end-point and those labelled with DNP–CGG and DNP–Fab also gave comparable titres.

Despite the fact that both methods of DNP coating the SRBC allowed the detection of equal numbers of PFC, the use of DNP–chicken anti-SRBC gamma-globulin-coated SRBC gave slightly, but consistently, lower affinity values for direct PFC than the use of DNP–Fab-coated SRBC—perhaps because DNP–chicken anti-SRBC coating produces red cells with a slightly higher number of DNP groups which cannot be detected by a difference in agglutinability.

The I_{50} values obtained showed little variation when the inhibition assay was performed with different batches of DNP-coated SRBC, but to ensure valid comparisons critical groups, e.g. 2 and 12-week-old mice in Table 1 and 1-, 2- and 12-week-old mice in Fig. 4 were assayed on the same day using identical DNP-coated SRBC.

RESULTS

In the first experiments, PFC found in the spleens of 2-week-old and 12-week-old (C57Bl × BALB/c)F1 mice 7 days after the injection of 200 μg of DNP–KLH were assayed and their relative affinities compared (Fig. 1). The I_{50} of PFC produced in 2-week-old mice was consistently higher than that of 12-week-old mice; that is, the affinity was lower. This difference was seen for both direct and indirect PFC.

Similar differences in the relative affinity of anti-DNP–PFC were observed in C57Bl and BALB/c mice (Table 1), therefore we felt justified in using the more readily available F1 hybrids in all subsequent experiments.

We considered that the apparently lower affinity of PFC in 2-week-old mice might be an artefact resulting from the attainment of a higher antigen concentration in the smaller animals so that their immune response failed to mature. The difference in weight between 2- and 12-week-old mice was about 5-fold. Consequently immunization over a range of doses was performed. The results (Table 2) indicated that the low affinity of anti-DNP–PFC in 2-week-old mice could not be explained on this basis. Variation in antigen does affect the relative affinity of PFC in 2-week-old mice (especially direct PFC). Nevertheless, comparison of relative affinities at 50 μg (for 2-week-old mice) and 200 μg (for 12-week-old mice) still shows a lower affinity in the 2-week-old mice for both direct and indirect PFC.

It was also possible that the maturation of affinity during the primary response in young mice was slower than in adults and that this accounted for the difference in relative

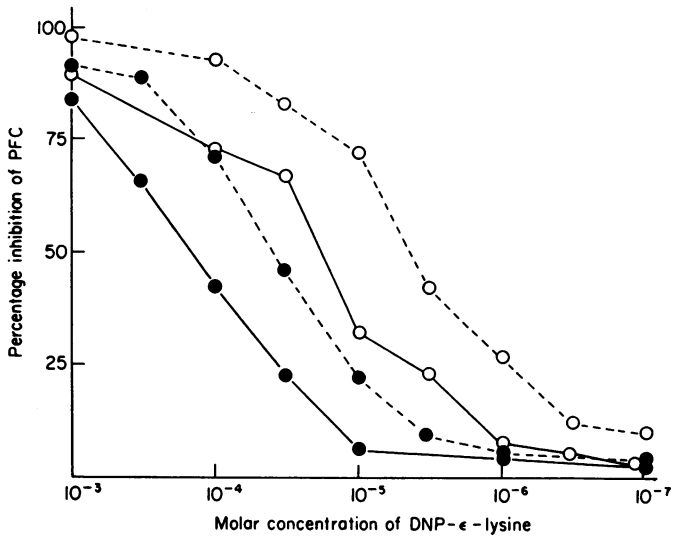

FIG. 1. Inhibition of anti-DNP–PFC in 2-week-old and 12-week-old (C57Bl×BALB/c)F1 mice by DNP–ε-lysine. (●) 2-week-old mice; (○) 12-week-old mice; (—) direct PFC; (- - -) indirect PFC. Each point represents the mean of four to twelve animals.

TABLE 1

RELATIVE AFFINITY AND NUMBERS OF ANTI-DNP–PFC IN 2- AND 12-WEEK-OLD BALB/c, C57Bl (C57Bl×BALB/c)F1 MICE

Strain	Age at immunization (weeks)	I_{50} (μmoles/l DNP–ε-lysine)		Anti-DNP PFC/spleen	
		Direct PFC*	Indirect PFC*	Direct PFC*	Indirect PFC*
BALB/c	2	42·8 (40·2–45·7)	16·7 (15·3–18·3)	31,070 (22,451–42,999)	36,623 (24,966–53,723)
BALB/c	12	16·2 (14·4–18·3)	2·6 (2·3–3·0)	33,833 (28,965–39,519)	66,769 (51,948–85,818)
C57Bl	2	62·0 (36·8–104·0)	31·1 (20·8–46·5)	2661 (1959–3615)	2576 (2005–3309)
C57Bl	12	17·9 (14·6–21·9)	3·6 (2·9–4·6)	32,919 (20,169–53,727)	55,834 (36,105–86,344)
(C57Bl×BALB/c)F1	2	53·8 (47·0–61·5)	33·5 (31·0–36·1)	32,009 (17,104–30,953)	24,072 (16,807–34,475)
(C57Bl×BALB/c)F1	12	12·2 (11·3–13·3)	3·4 (3·0±3·8)	49,891 (35,148–49,927)	83,320 (66,938–103,711)

The results are expressed as the geometric mean (± one standard error). Four to twelve mice were used per group.
* Assayed on DNP–Fab-coated SRBC, 7 days after 200 μg of DNP–KLH.

affinities measured on day 7. Determination of the I_{50} of anti-DNP–PFC at various times after immunization in the two groups showed no significant difference in the rate of increase of affinity between them. When maturation was occurring it was equally rapid in both the young and the adult mice (Fig. 2). It is noteworthy that our data show a slight maturation of affinity in the direct PFC for both 2- and 12-week-old mice. This is in agreement with the findings of Wu and Cinader (1972), although there is conflicting evidence in the literature, e.g. Huchet and Feldmann (1973). In our case we have no

formal proof that all direct PFC are IgM secretors since inhibition with anti-μ serum has not been attempted.

A third possible explanation of the lower affinity of PFC in the 2-week-old mice was that they had a functional deficiency of T cells, and that these cells are required for the expression of high affinity PFC. To test this, 2×10^7 'B' spleen cells, irradiated 'B' spleen cells, or adult thymus cells were injected into 12-day-old mice and the recipients challenged 2 days

TABLE 2

RELATIVE AFFINITY (A) AND NUMBERS (B) OF ANTI-DNP–PFC IN 2- AND 12-WEEK-OLD (C57Bl × BALB/c)F1 MICE FOLLOWING VARIOUS DOSES OF DNP–KLH

A Dose of DNP–KLH (μg)	I_{50} (μmoles/l DNP–ε-lysine)				
	2-week-old*		12-week-old*		12-week-old
	Direct PFC	Indirect PFC	Direct PFC	Indirect PFC	Indirect PFC
400	490 (330–740)	57·8 (46·7–71·5)	n.d.	n.d.	n.d.
200	135 (110–160)	35·7 (32·0–39·8)	23·8 (17·4–32·7)	3·2 (2·9–3·7)	n.d.
100	320 (260–380)	57·1 (40·2–81·0)	41·5 (29·6–58·2)	1·7 (1·2–2·3)	0·18 (0·11–0·29)
50	117 (89–150)	27·1 (22·5–32·6)	n.d.	n.d.	n.d.
20	n.d.	n.d.	32·9 (27·3–39·7)	2·6 (2·4–2·9)	n.d.
B	**Anti-DNP PFC per spleen**				
	2-week-old*		12-week-old*		12-week-old†
	Direct PFC	Indirect PFC	Direct PFC	Indirect PFC	Indirect PFC
400	83,724* (741,42–94,544)	161,912 (132,115–198,430)	n.d.	n.d.	n.d.
200	24,626 (17,554–34,555)	37,513 (24,405–57,661)	55,065 (46,569–65,109)	68,906 (41,384–114,730)	n.d. n.d.
100	14,434 (13,439–15,503)	20,177 (18,878–21,566)	15,997 (11,489–22,275)	53,968 (45,474–64,048)	344,080 (302,914–390,842)
50	26,264 (19,410–35,266)	38,728 (29,725–50,459)	n.d.	n.d.	n.d.
20	n.d.	n.d.	10,992 (9,278–13,023)	11,945 (8,768–16,274)	n.d.

n.d. = Not determined.

The results were expressed as geometric mean (± one standard error). Four to eight mice were used per group.

* Immunized 7 days previously with stated antigen dose.

† Challenged 5 days previously with stated dose; 8 weeks after priming with 100 μg of DNP–KLH.

later with 200 μg of DNP–KLH. Seven days later the relative affinities of anti-DNP–PFC in the various groups were assessed. The results of this experiment (Table 3) showed that the 'adult' 'B' spleen cells were quite capable of expressing their higher average affinity even when co-operating with the T cells of the 2-week-old mice. A higher average affinity of PFC was not detected after the transfer of adult thymocytes, arguing against a functional inadequacy of young T cells. The possibility that the high mean I_{50} normally seen in the young mice resulted from environmental factors such as hormones could also be

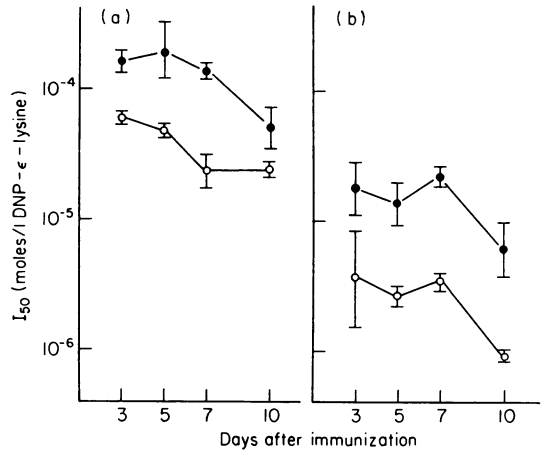

FIG. 2. Relative affinity of (a) direct and (b) indirect anti-DNP–PFC at various times after immunization in 2- and 12-week-old mice. (●) 2-week-old mice; (○) 12-week-old mice. Each point represents the geometric mean of four to twelve animals ± the standard error of the mean. All mice were immunized with 200 μg of DNP–KLH.

TABLE 3

THE EFFECT OF TRANSFERRING 'B' SPLEEN OR THYMUS CELLS INTO NORMAL SYGENEIC MICE ON THE RELATIVE AFFINITY AND NUMBERS OF ANTI-DNP–PFC

Group*	Age at immunization (weeks)	Cells transferred†	I_{50}‡ (μ moles/l DNP–ε-lysine)	Indirect anti-DNP–PFC per spleen‡
A	2	None	32·7 (29·0–36·9)	23,937 (13,478–42,512)
B	12	None	2·8 (2·2–3·5)	34,038 (21,037–55,071)
C	2	'B' spleen	4·4 (2·6–7·8)	29,725 (21,488–41,120)
D	2	Irradiated 'B' spleen	38·9 (33·0–45·9)	26,107 (21,847–31,199)
E	2	Adult thymus	24·6 (20·3–29·8)	31,566 (16,655–59,824)
F	12	'B' spleen	2·3 (1·9–2·9)	109,105 (72,322–164,595)

The results are expressed as geometric mean (± one standard error). Five mice were used per group.

* A *vs* C, $P < 0{\cdot}0005$; B *vs* C, $P = 0{\cdot}25$, by Student's *t*-test.
† 2×10^7 cells given intraperitoneally 2 days before antigen.
‡ Measured on indirect PFC 7 days after 200 μg of DNP–KLH.

excluded. This point is reinforced by the finding that on transfer to lethally irradiated adults, 2-week-old spleen cells still gave, on challenge, PFC with characteristic low affinity (Table 4). In this latter situation one would expect to have the relatively radio-resistant macrophages of the adult recipient participating in the response, so it seems unlikely that the low affinity of anti-DNP–PFC in the 2-week-old mice is a result of an 'immature' antigen-processing system.

The most plausible explanation of the difference in relative affinity thus seemed to be

TABLE 4

RELATIVE AFFINITY AND NUMBERS OF ANTI-DNP–PFC IN IRRADIATED ADULT C57Bl × BALB/c)F1 MICE RECEIVING 2- OR 12-WEEK OLD SYNGENEIC SPLEEN CELLS

	I_{50} (μmoles/l DNP–ε-lysine)		Anti-DNP–PFC per spleen	
Cells transferred*	Direct PFC†	Indirect PFC†	Direct PFC†	Indirect PFC†
2-week-old spleen	42·7 (39·9–45·9)	20·2 (18·7–21·9)	7917 (6007–9434)	5109 (3983–6553)
12-week-old spleen	10·6 (7·9–14·2)	2·1 (1·5–2·8)	9640 (7942–11,702)	4882 (4268–5662)

The results were expressed as geometric mean (± one standard error). Four mice were used per group).

* 5×10^7 cells transferred on the day of irradiation (850 rad) and immunization.

† Assayed on DNP–Fab-coated SRBC, 8 days after 200 μg of DNP–KLH.

that progressive selection of higher affinity PFC precursors by environmental antigen was occurring during ontogeny.

Determination of the relative affinity of anti-DNP–PFC of mice aged 1–24 weeks (Fig. 3) shows that during the first 6 weeks of life there is at least a 10-fold decrease in the I_{50} for indirect PFC and an 8- to 10-fold decrease for direct PFC. By 6 weeks of age the affinity of PFC had increased to a level that remained constant at least up to 24 weeks of age. This level did not, however, reflect either the limit of maturation of the response to DNP–KLH or any limit on the detection of high affinity PFC by the assay, since 12-week-old mice primed with DNP–KLH and rechallenged 8 weeks later gave PFC with an I_{50} of $1{\cdot}8 \times 10^{-7}$ M indicating a relative affinity higher than any seen in the primary responses (Table 1).

By comparing the number of PFC inhibited over separate concentration ranges of DNP–ε-lysine, it is possible to analyse the distribution of their affinities (Davie and Paul, 1972). Such an analysis (Fig. 4) shows that in young mice there is a lack of those cells inhibited by low concentrations of DNP–ε-lysine which is progressively corrected with

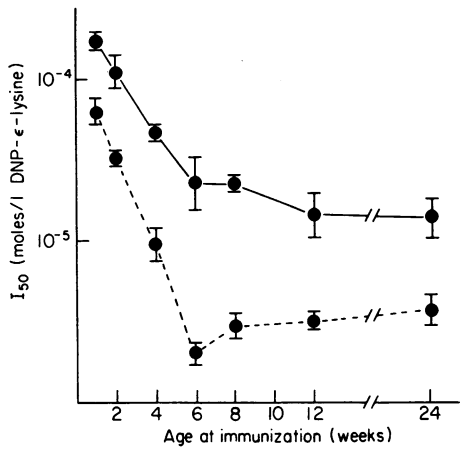

FIG. 3. Age-dependent change in the relative affinity of anti-DNP–PFC. (—) direct PFC; (– – –) indirect PFC. Each point represents the geometric mean of four to twelve animals ± the standard error of the mean. All mice received 200 μg of DNP–KLH 7 days previously.

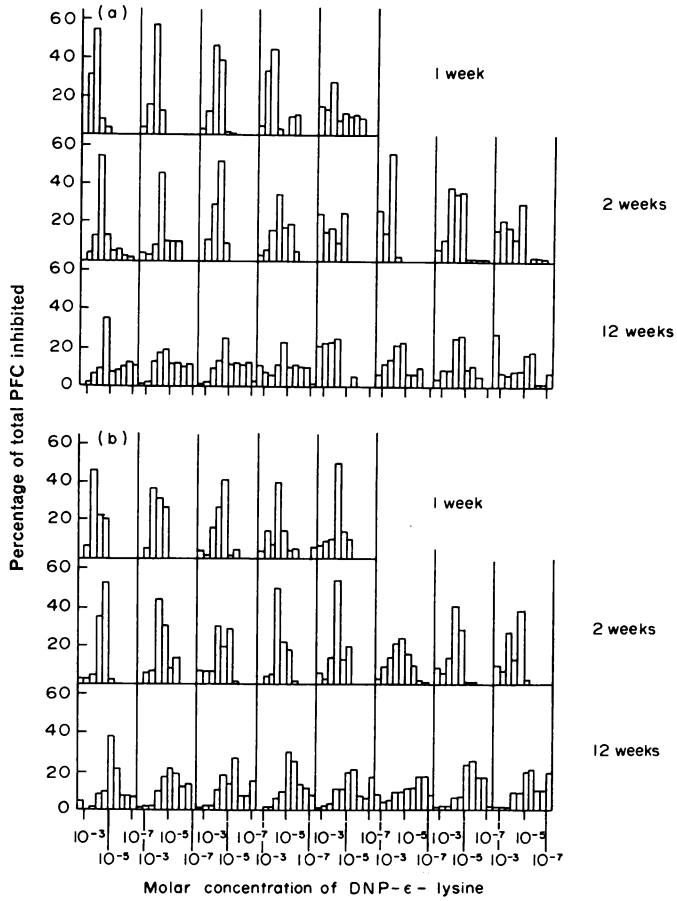

FIG. 4. The affinity profiles of anti-DNP–PFC in 1-, 2- and 12-week-old (C57Bl × BALB/c)F1 mice 7 days after 200 μg of DNP–KLH. (a) Direct PFC. (b) Indirect PFC.

age, and that those PFC only inhibited at high concentrations become a less predominant feature of the population during this time.

DISCUSSION

The data presented here indicate that during murine development there occurs a marked change in the affinity of anti-DNP antibodies as measured at the cellular level. This increase is not a result of the inability of T cells in neonatal mice to co-operate with high affinity B cells, but rather is due to a change in the B-cell population itself. This change might be attributable to the selection of antibody-forming precursor cells of progressively higher affinity by environmental antigen. We propose to study this possibility by the use of germ-free animals.

From these findings it would be expected that mice immunized in the early post-natal period should show optimal antibody responses at higher antigen doses than mice injected in adulthood, because of the predominance of low affinity precursors in early life. This

expectation is borne out in a recent paper by Rector and Carter (1973). These workers found that the sensitivity of mice to SRBC is age-dependent, whilst 12-day-old animals responded only over a narrow range of doses, $3{\cdot}8 \times 10^9$ SRBC producing an optimal IgG2a response, 12-week-old animals reacted to a wider range with an optimum dose of $1{\cdot}0 \times 10^8$ for an IgG2a response. They attribute this difference to either progressive selection of receptors by environmental antigen or to age-dependent changes in the antigen-processing system. Their use of a complex multi-determinant antigen unfortunately precluded the direct measurement of antibody affinity at the cellular level.

Contrasting results have been obtained by Press and Klinman (1973) who demonstrated that the dose dependence of *in vitro* stimulation by the DNP group on KLH was similar for splenic antibody-forming foci derived from both adult and neonatal tissues.

The studies reported here provide evidence of restriction of heterogeneity of the anti-DNP response in neonatal mice, and the possibility of responses of restricted clonality is being pursued by analysing the iso-electric spectra of anti-DNP antibodies (Williamson, 1971). By the use of this method restricted responses have been found in neonatal rabbits (Montgomery and Williamson, 1972). Our preliminary data indicate that this is also true for the primary response to DNP–KLH in mice.

Somatic mutation theories of antibody diversity (Cunningham and Pilarski, 1974) predict that foetal and neonatal animals should possess a restricted number of clones of antibody-forming cell precursors which one would expect to have receptors of low affinity for a given antigen. Cells bearing receptors of new specificity, including some of higher affinity for the given antigen, would then arise during ontogeny as the result of mutation.

Our data are clearly consistent with these predictions. However, we cannot rule out the possibility that the increase of affinity during ontogeny is due to selection alone, as has been postulated to occur during the immune response (Siskind and Benaceraff, 1969): environmental antigen may be selecting a population of cells bearing high affinity (germ-line encoded) receptors which are present in young mice at a level that we are unable to detect.

During the preparation of this manuscript Goidl and Siskind (1974), using a system in which murine B cells taken at various stages during ontogeny were transferred with an excess of adult T cells to irradiated recipients, have reported results which are in close agreement with ours.

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