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Deficient antibody formation in the bone marrow of nude mice

R. BENNER, A. VAN OUDENAREN & J. J. HAAIJMAN* *Department of Cell Biology and Genetics, Erasmus University, P.O. Box* 1738, *Rotterdam and* **Institute for Experimental Gerontology TNO,* 151 *Lange Kleiweg, Rijswijk (ZH), The Netherlands*

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Summary. The bone marrow of young adult nude mice was investigated as a site of antibody formation after intravenous immunization with the thymus-independent antigen *Escherichia coli* lipopolysaccharide (LPS). Mice heterozygous for the nu-gene were found to be capable of a plaque-forming cell (PFC) response in both spleen and bone marrow after primary and secondary immunization with LPS. Primary immunization of nude mice with LPS induced a normal PFC response in the spleen, but did not evoke the appearance of PFC in the bone marrow. During the secondary response the nude mice did show PFC activity in the bone marrow, but at a much lower level than their heterozygous littermates. At all time points after secondary immunization the number of splenic PFC was higher in nude mice than in the control mice.

Determination by immunofluorescence of cells containing cytoplasmic immunoglobulin (C-Ig cells) in the bone marrow of young adult nonimmune nude and heterozygous mice, revealed a three times higher number of C-IgM cells in the bone marrow of the heterozygous thymus-bearing mice. On the other hand, the number of splenic C-IgM cells was higher in the nude mice than in their heterozygous littermates. These results suggest that the presence of the thymus facilitates the appearance of mature antibody-forming cells in the bone marrow of young adult mice, irrespective of whether the generation of these cells is initiated by so called thymus-dependent or thymus-independent antigens.

Correspondence: Dr R. Benner, Department of Cell Biology and Genetics, Erasmus University, P.O. Box 1738, Rotterdam, The Netherlands.

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INTRODUCTION

The bone marrow is an important site of antibody production in mice. This follows from a number of studies using both thymus-dependent (Benner, Meima, Van der Meulen & Van Muiswinkel, 1974a; Benner, Meima, Van der Meulen & Van Ewijk, 1974b; Hill, 1976) and thymus-independent antigens (Benner & Van Oudenaren, 1976; 1977). After primary immunization with thymus-dependent antigens only minor numbers of antibody-producing plaque-forming cells (PFC) are to be found in the bone marrow (Benner *et al.*, 1974a; Hill, 1976; Eidinger & Pross, 1967; Chaperon, Selner & Claman, 1968; Mellbye, 1971; Anderson & Dresser, 1972; Cohen, 1972). After a second injection of the same antigen, however, the number of PFC in the bone marrow can rise to a level which surpasses the total number of PFC in all the other lymphoid organs together (Benner *et al.*, 1974a; 1974b; Hill, 1976). After an intravenous booster injection of a moderate dose of antigen, the spleen is involved in the initiation of the bone marrow response. This spleen involvement follows from experiments with parabiotic mice, and from experiments in which

animals were splenectomized at various intervals after the booster injection (Benner, Van Oudenaren & De Ruiter, 1977).

Immunization with the thymus-independent (Möller & Michael, 1971; Andersson & Blomgren, 1971) antigen *Escherichia coli* lipopolysaccharide (LPS) evokes the appearance of large numbers of anti-LPS PFC in the bone marrow, not only during the secondary, but also during the primary response (Benner & Van Oudenaren, 1976; 1977). This might be attributed to the nonrequirement for T cell help in the response to thymus-independent antigens, so that the B cells in the bone marrow can respond with a PFC response directly upon the first encounter with LPS (Benner & Van Oudenaren, 1976). This hypothesis implies that the response in the bone marrow after primary injection of mice with LPS should not be affected by T cell deprivation. This prediction was tested in the present paper by using young adult, congenitally athymic nude (nu/nu) mice and their heterozygous (nu/+), thymus-bearing, littermates. The results presented in this paper show that the presence of the thymus is of utmost importance for a normal anti-LPS PFC response in the bone marrow, while the thymus is not required for the splenic PFC response to LPS. Determination of the numbers of cells containing cytoplasmic immunoglobulins (C-Ig cells) in the bone marrow of nu/nu and nu/+ mice, revealed low numbers of C-Ig cells in the bone marrow of nu/nu mice. These results suggest that the overall antibody formation is deficient in the bone marrow of young adult nude mice.

MATERIALS AND METHODS

Mice

Specific pathogen free nude mice and their heterozygous littermates (8–10 weeks old) on a B10.LP background were purchased from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands. (C57BL/Rij × CBA/Rij) F1 female mice, 6 weeks old, were purchased from the Medical Biological Laboratory, Rijswijk, The Netherlands. Pelleted food (Hope Farms, Woerden, The Netherlands) and acidified water (pH 3–4) were given *ad libitum*.

T cell-depleted mice

(C57BL/Rij × CBA/Rij) F1 female mice were depleted of T cells by adult thymectomy at 6 weeks of age, followed by lethal irradiation (925 rad) and reconstitution with 2×10^6 syngeneic foetal liver cells i.v. (Rozing, Brons & Benner, 1977) 3 weeks later. Control mice were sham-thymectomized, and otherwise similarly treated. The mice were used in the experiments 3 months after irradiation and reconstitution.

Antigen and immunization

LPS from *E. coli* 055 : B5, prepared according to the phenol-extraction method (Westphal, Lüderlitz & Bister, 1952), was obtained from Difco Laboratories, Michigan. It was dissolved in a balanced salt solution (BSS), prepared according to Mishell & Dutton (1967). Mice were immunized by i.v. injection of doses of LPS ranging from 0·001 μg–10 μg in 0·5 ml BSS. For secondary immunization mice received a booster injection 6 weeks after the first.

Preparation of cell suspensions

Mice were killed by carbon dioxide. Immediately after killing, spleens and femurs were removed, and single cell suspensions were prepared as described previously (Benner *et al.*, 1974a). Nucleated cells were counted with a Coulter counter Model B.

Plaque-forming cell (PFC) assay

Anti-LPS PFC were determined as described in detail in a previous paper (Benner & Van Oudenaren, 1976). Bone marrow PFC activity was determined in cell suspensions obtained from the femoral marrow. The number of PFC present in the bone marrow of the whole animal was estimated from the data of the femoral bone marrow, using the data of Chervenick and co-workers (Chervenick, Boggs, Marsh, Cartwright & Wintrobe, 1968), who showed that in mice one femur contains 5·9% of the total marrow. Previously no differences could be detected between the PFC responses evoked by equal numbers of nucleated bone marrow cells from femur, tibia, humerus, rib and sternum, which indicated that the PFC activity in various compartments of mouse bone marrow is about the same (Benner & Van Oudenaren, 1976).

Passive haemagglutination titres

Anti-LPS antibody titres in serum were assayed by means of a passive haemagglutination technique using a 1% suspension of SRBC coated with LPS. Details of the procedure have been described previously (Benner & Van Oudenaren, 1976).

Assay for cells containing cytoplasmic immunoglobulin (C-Ig cells)

Cell suspensions to be tested for C-Ig cells were spun down (10 min, 1500 rev/min) and resuspended in phosphate buffered saline (PBS) supplemented with 5% bovine serum albumin (BSA; Poviet, Amsterdam, The Netherlands) and 0·1% EDTA (Titriplex, Merck A.G., Darmstadt, Western Germany). Properly adjusted cell suspensions containing a known number of nucleated cells were spun down in a cytocentrifuge according to Vossen (1975). C-Ig cells were visualized as described in detail by Hijmans, Schuit & Klein (1969). All immunocytes positive for cytoplasmic Ig were counted per cytocentrifuge slide. The slightly positive small lymphocytes, most likely bearing membrane bound IgM, were excluded. The total number of positive cells per organ was calculated using the number of nucleated cells per slide and the total cell yield of a given organ. A goat antiserum directed against mouse immunoglobulin, and conjugated with fluorescein isothiocyanate (GAM/Ig-FITC; Nordic Immunological Laboratories, Tilburg, The Netherlands) was applied for the total C-Ig cell count. The class distribution was determined according to Hijmans *et al.* (1969), using antisera specific for the Fc part of the different mouse immunoglobulins. These antisera were conjugated with either tetramethyl rhodamine isothiocyanate (TRITC) or FITC. The antisera met all specificity criteria described in detail in a previous paper (Haaijman, Schuit & Hijmans, 1977).

The slides were examined with either a Zeiss Standard microscope equipped with an ep-illuminator IV/F and an Osram HBO 50 mercury lamp, or a Zeiss Universal microscope equipped for epi-illumination and an Osram HBO 100 mercury lamp. Filter combinations for both microscopes have been described previously (Haaijman *et al.*, 1977; Van der Ham, Benner & Vos, 1977).

RESULTS

PFC response after immunization with LPS

The primary response to LPS was studied in 8–10 weeks old nude mice and their heterozygous littermates after i.v. injection of 5 μg of the antigen. PFC were determined in spleen and bone marrow of both groups of mice at various times after immunization. The first PFC were detectable in the spleen of nu/nu and nu/+ mice on the 3rd day after immunization (Fig. 1). Peak PFC responses were found in the spleen on day 5. Thereafter the splenic PFC response steadily declined. Anti-LPS PFC activity in the bone marrow could only be demonstrated in nu/+ mice, showing the peak response on day 5. In the bone marrow of nu/nu mice no significant PFC activity (less than 500 PFC per whole bone marrow) was observed at any time after immunization.

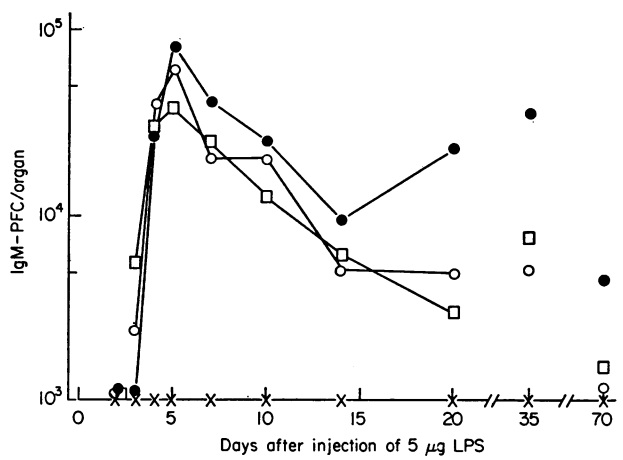

Figure 1. Number of PFC in spleen and bone marrow of nu/+ and nu/nu mice immunized with 5 μg LPS i.v. (○) Spleen and (●) bone marrow of nu/+ mice. (□) Spleen of nu/nu mice. In the bone marrow (×) of nu/nu mice no significant PFC response was found at any time after immunization. Each group consisted of 4–5 mice. PFC were assayed in pooled cell suspensions.

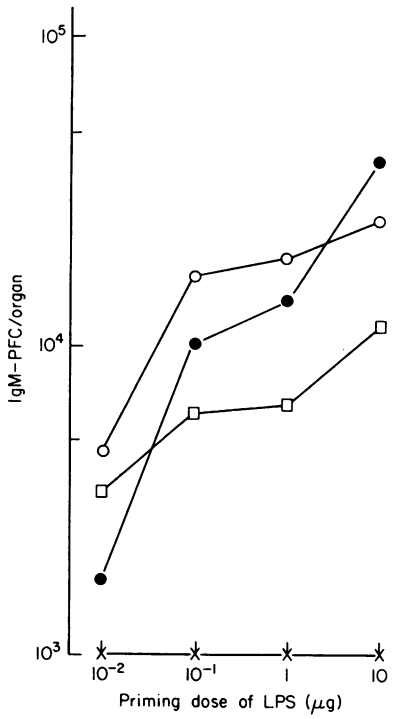

Figure 2. Number of PFC in spleen and bone marrow of nu/+ and nu/nu mice 6 days after immunization with either 0·01, 0·1, 1 or 10 μg LPS i.v. (○) Spleen and (●) bone marrow of nu/+ mice. (□) Spleen of nu/nu mice. In the bone marrow (×) of nu/nu mice no significant PFC response was found after either dose of priming. Each group consisted of 5 mice. PFC were assayed in pooled cell suspensions.

This absence of bone marrow PFC activity in nu/nu mice might be due to the rather high antigen dose of 5 μg LPS. Doses of 5 μg LPS and higher frequently induce granulopoiesis in the bone marrow of nu/nu mice (data not shown), and this might interfere with the PFC response *in situ*. In nu/+ mice this effect of LPS is only found after higher doses. Therefore, we also studied the PFC response in nu/nu and nu/+ mice after 0·01, 0·1, 1 and 10 μg LPS i.v.

Determination of the PFC responses 6 days after immunization did not reveal PFC activity in the marrow of the nu/nu mice at any dose of LPS (Fig. 2). The nu/+ mice, on the other hand, showed increasing bone marrow PFC responses with increasing doses of LPS. Similar data were obtained in thymectomized compared to sham-thymectomized, lethally irradiated, foetal liver reconstituted mice. Also in this case the T cell deprived mice showed a deficient anti-LPS bone marrow PFC response (Table 1).

The secondary response to LPS was studied in nu/nu and nu/+ mice primed with 1 μg LPS i.v., and boosted with 5 μg LPS i.v. 6 weeks later. In this case not only the nu/+, but also the nu/nu mice reacted with a PFC response in both spleen and bone marrow (Fig. 3). In both groups of mice the splenic PFC responses were some times higher than after primary injection of 5 μg LPS i.v. Remarkably enough, the secondary PFC responses in the spleen of the nu/nu mice were higher than in the spleen of

Table 1. PFC response of T cell deprived (C57BL × CBA) F1 mice to LPS*

Organ	Dose of LPS (μg)	PFC/Organ	
		Tx.FL mice	SHTx.FL mice
Spleen	1	11,280† (8890–14,310)	11,860 (8750–16,080)
Bone marrow	1	< 500	10,170 (7880–13,130)
Spleen	10	18,850 (15,800–22,490)	20,660 (11,970–16,080)
Bone marrow	10	2490 (1520–4030)	45,270 (40,090–51,120)

* Adult thymectomized (ATx.FL) and sham-thymectomized (SHTx.FL) mice were lethally irradiated and reconstituted with 2×10^6 foetal liver cells 3 weeks later. Three months after irradiation and reconstitution the mice of both groups were immunized with either 1 or 10 μg LPS i.v. PFC assay was done 6 days after immunization.

† Geometric mean ± 1 SEM of 5 mice.

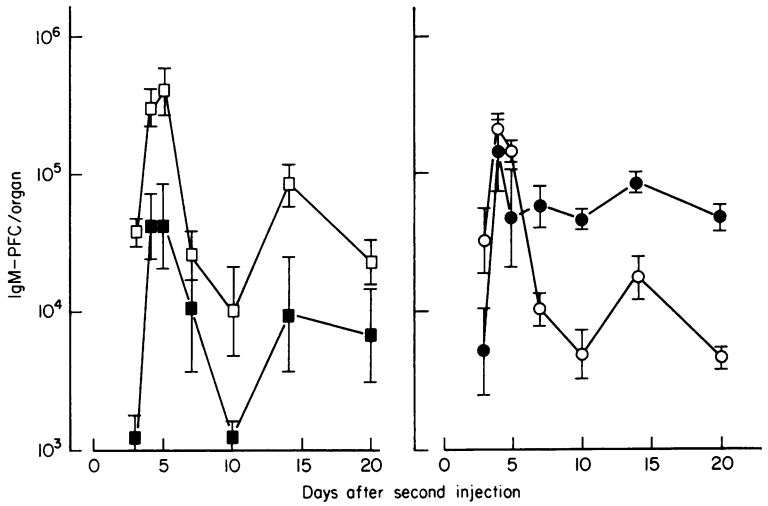

Figure 3. Number of PFC in spleen and bone marrow of nu/+ and nu/nu mice primed with 1 μg LPS i.v. and boosted with 5 μg LPS i.v. 6 weeks later. (○) Spleen and (●) bone marrow of nu/+ mice. (□) Spleen and (■) bone marrow of nu/nu mice. The mice were tested individually. Each point represents the geometric mean ± 1 SEM of 4–5 mice.

Table 2. Passive haemagglutination titre in nude mice and other T cell deprived mice after immunization with LPS

Mice	Primary response*		Secondary response†	
	Before priming	After priming	Before boosting	After boosting
nu/nu	1·24‡ (1·07–1·44)	5·00 (4·09–6·12)	2·72 (2·08–3·54)	10·56 (9·40–11·87)
nu/+	2·53 (2·15–2·98)	8·49 (7·17–10·07)	4·31 (3·41–5·44)	9·71 (8·77–10·75)
Tx.FL§	1·56 (1·31–1·86)	8·34 (7·73–8·99)	n.d.¶	n.d.
SHTx.FL§	1·62 (1·39–1·88)	11·11 (10·48–11·78)	n.d.	n.d.

* Mice were immunized with 10 μg LPS i.v.

† Mice were primed with 1 μg LPS i.v. and boosted with 5 μg LPS i.v. 6 weeks later.

‡ Geometric mean ± 1 SEM of the $\log_2$ antibody titre of 5 mice. Sera were collected just before and 6 days after the first or second injection of LPS.

§ Tx.FL = adult thymectomized, lethally irradiated and foetal liver reconstituted mice. SHTx.FL = adult sham-thymectomized, lethally irradiated, foetal liver reconstituted mice.

¶ n.d. = not determined.

the nu/+ mice. On the other hand, the bone marrow PFC responses were higher in the nu/+ than in the nu/nu mice. Furthermore, the variation between the bone marrow PFC responses of individual nu/nu mice was larger than between those of nu/+ mice.

The passive haemagglutination titres in the sera of nu/nu and nu/+ mice 6 days after primary and secondary immunization with LPS reflected the PFC responses in spleen and bone marrow during the foregoing days. After primary immunization the titres were lower in the nu/nu mice, probably due to the absence of antibody formation in the bone marrow (Table 2). A similar difference was found between thymectomized and sham-thymectomized, irradiated, and foetal liver reconstituted mice. Such a difference was not found 6 days after a booster injection of 5 μg LPS. In contrast to the primary response, the increase in anti-LPS antibody

Table 3. Cells containing cytoplasmic immunoglobulins (C-Ig cells) in spleen and bone marrow of nonimmune nude mice ($\times 10^{-3}$)

	Spleen				Bone marrow†			
Mice*	Total C-Ig	C-IgM	C-IgG	C-IgA	Total C-Ig	C-IgM	C-IgG	C-IgA
nu/nu	137 ± 17‡	95§	2·4	2·9	54 ± 7	30	0·9	1·8
nu/+	130 ± 23	34	19	32	292 ± 47	93	84	96

* The nu/nu and nu/+ mice used in this experiment were 8 weeks old.

† Figures represent the number of C-Ig cells as calculated for the total bone marrow of the animal.

‡ Arithmetic mean ± 1 SEM of 5–7 mice.

§ Figures represent the number of C-Ig cells positive for only one heavy chain class. The difference between the number of total C-Ig cells and the sum of the C-IgM, C-IgG and C-IgA cells gives the number of C-Ig cells positive for more than one heavy chain class.

titre was smaller in nu/+ mice than in nu/nu mice. This is in harmony with the high PFC activity in the spleen of the nu/nu mice. During the early phase of the secondary response in nu/nu mice the spleen amply compensated for the low response in the marrow (Fig. 3).

Distribution of cells containing cytoplasmic immunoglobulins

The numbers of cells containing cytoplasmic immunoglobulins (C-Ig cells) were quantified in spleen and bone marrow of 8 weeks old nu/nu and nu/+ mice by means of an immunofluorescence technique. The total number of C-Ig cells per spleen appeared to be the same for nu/nu and nu/+ mice (Table 3). On the other hand, the number of C-Ig cells in the bone marrow of the same mice was clearly different. The number of C-Ig cells in the total bone marrow of nu/nu mice was calculated to be 48,000, whereas this figure for nu/+ mice was 292,000. The class distribution of the C-Ig cells was the most striking difference between nu/nu and nu/+ mice. Both in spleen and bone marrow of the nu/nu mice the larger part of the C-Ig cells contained IgM (Table 3). On the contrary, a large proportion of the C-Ig cells in the nu/+ mice proved to be positive for IgG and IgA.

DISCUSSION

Phenol-extracted LPS from the *E. coli* strain 055 : B5 evokes splenic PFC responses and serum antibody titres of about the same magnitude in T cell deprived and normal control mice (Fig. 1; Tables 1 and 2; Andersson & Blomgren, 1971; Möller & Michael, 1971). These observations have previously led to the conclusion that in mice this particular preparation of LPS is a thymus-independent antigen. However, the data presented in this paper show that, as far as antibody formation in the bone marrow is concerned, T cells facilitate antibody formation to this antigen (Figs 1–3; Table 1). This should have implications for the definition of antigens as being thymus-independent. In our opinion it is important to mention in such a definition the aspect which is studied, and the assays which are used. In this sense, LPS is in mice a thymus-independent antigen as far as it concerns antibody formation *in the spleen*, but thymus-dependent as regards bone marrow antibody formation. In the literature more evidence can be found, that different processes induced by one and the same 'thymus-independent' antigen can differ in their requirement for T cell functions (Kagnoff, Billings & Cohn, 1974; Mond, Corporale & Thorbecke, 1974; Ness, Smith, Talcott & Grumet, 1976).

A booster injection of nu/nu mice with LPS induced a typical secondary type PFC response in the spleen (cf. Figs 1 and 3). Comparison of the secondary anti-LPS response in nu/nu and nu/+ mice shows that priming of nu/nu mice with LPS induces B cell memory. Apparently T cells are not required for the generation of B memory cells specific for LPS. Although similar results have been reported by others using different antigens (Roelants & Askonas, 1972; Mond *et al.*, 1974; Diamantstein & Blitstein-Willinger, 1974; Schrader, 1975), the relationship between T cells and the generation of B memory cells is still a matter of controversy (Braley-Mullen, 1976; Okumura, Metzler, Tsu, Herzenberg & Herzenberg, 1976; Klaus, 1977).

In contrast to primary immunization, secondary immunization of nu/nu mice with LPS did evoke PFC activity in both spleen and bone marrow (Fig. 3). However, the kinetics of the secondary anti-LPS PFC response in nu/nu and nu/+ mice was different. At all times after booster injection the numbers of PFC in the spleen of nu/nu mice were higher than those in nu/+ mice. On the other hand, bone marrow PFC responses were always higher in nu/+ mice. Apparently, in the absence of the thymus and T cells the normal balance between splenic and bone marrow anti-LPS PFC activity is disturbed. So far there is no conclusive evidence available whether the appearance of anti-LPS PFC in the bone marrow is due to activation of B cells by LPS within the marrow, or to activation of B cells by LPS in the spleen, and subsequent migration of these activated B cells towards the bone marrow, where the maturation into PFC occurs (Benner & Van Oudenaren, 1977). Therefore, at present it is difficult to analyse the exact role of T cells in the process of bone marrow antibody formation. Maybe the clue to the solution of the problem lies in the observation of Baker, Stashak, Amsbaugh & Prescott (1971) that immunization of mice with optimal doses of type III pneumococcal polysaccharide (SIII) does not induce the appearance of PFC in the bone marrow. The SIII antigen does not contain a component with the properties of lipid A, which normally occurs in LPS (Von Eschen & Rudbach, 1976). This lipid A is a.o. responsible for the adjuvant and mitogenic activities of LPS (Kagnoff *et al.*, 1974; Von Eschen & Rudbach, 1976; Kolb, Di Pauli & Weiler, 1976). A number of recent reports have shown that T cells are required for the expression of the lipid A mediated effects of LPS (Armerding & Katz, 1974; Kolb *et al.*, 1976; Scibienski & Gershwin, 1977). Involvement of a component like lipid A in bone marrow antibody formation to thymus-independent antigens, would therefore explain the deficient antibody formation in the marrow of nu/nu mice immunized with LPS. It would also imply a deficient antibody formation in the bone marrow of normal mice immunized with any one thymus-independent antigen lacking a component with the properties of lipid A. In this respect, it would be of great interest to investigate whether detoxification of LPS, which inactivates the lipid A component (Von Eschen & Rudbach, 1976), can prevent the appearance of PFC in the bone marrow of normal mice.

The deficient antibody formation in the bone marrow of young adult nu/nu mice, as found after immunization with LPS, is a general phenomenon, directly related to the low incidence of T cells in these mice. This is apparent from the low numbers of C-Ig cells in the bone marrow of nu/nu mice, as compared with nu/+ mice (Table 3). Since the overall IgG and IgA antibody production are more dependent on T cells than the IgM response (Pritchard, Riddaway & Micklem, 1973; Bloemmen & Eyssen, 1973; Van Muiswinkel & Van Soest, 1975), only low numbers of C-IgG and C-IgA cells are found in nu/nu mice (Table 3). In the spleen this is compensated for by increased numbers of C-IgM cells, yielding a total C-Ig count comparable to nu/+ mice. However, in the marrow of nu/nu mice the number of C-IgM cells is much lower than in nu/+ mice. This suggests that in young adult nu/nu mice the IgM synthesis is restricted mainly to the spleen, and that bone marrow antibody formation is facilitated by T cells.

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