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Antibody formation in mouse bone marrow

V. THE RESPONSE TO THE THYMUS-INDEPENDENT ANTIGEN *ECSHERICHIA COLI* LIPOPOLYSACCHARIDE

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Summary. The occurrence of plaque-forming cells (PFC) in mouse bone marrow was studied during primary and secondary responses to the thymus-independent antigen *Escherichia coli* lipopolysaccharide (LPS). Anti-LPS responses were induced by various doses of LPS.

During the primary response, doses of 1 and 10 μg LPS intravenously (i.v.) were found to evoke a distinct PFC response in both spleen and bone marrow. The spleen contained the majority of PFC until about 5 days after immunization. During the course of the reaction the number of PFC in the bone marrow rose to a level which equalled or surpassed the level in the spleen. LPS doses of 0·001, 0·01 and 0·1 μg i.v. only induced a PFC response in the spleen. Apparently there is a minimal threshold dose of LPS of about 1 μg for PFC to appear in the bone marrow.

The secondary response was studied in mice primed with 1 μg LPS i.v. and boosted with either 0·001, 0·1 or 10 μg LPS i.v. 3 months later. After each dose tested the PFC activity in the spleen was several times higher than during the primary response. As was observed in the primary response doses of 0·001 and 0·1 μg LPS i.v. did not evoke a PFC response in the bone marrow. After boosting with 10 μg of LPS i.v. a significant PFC response was found in spleen, bone marrow, thymus, lymph nodes, Peyer's patches and blood. From about 5 days after the booster injection the number of PFC in the bone marrow exceeded the total number found in all other lymphoid organs. The results are discussed in relation to the bone marrow PFC response to the thymus-dependent antigen sheep red blood cells. To this antigen a clear PFC response in the bone marrow is found only during the secondary response.

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INTRODUCTION

Previous papers in this series on antibody formation in mouse bone marrow dealt with the plaque-forming cell (PFC) response to the thymus-dependent sheep red blood cell antigen (SRBC). After the second injection of SRBC, but not after the first, a very distinct IgM–, IgG– and IgA–PFC activity was found in the bone marrow (Benner, Meima, van der Meulen and van Muiswinkel, 1974d). Independent of the booster dose this activity in the bone marrow appeared to rise to a level which surpassed the total level of all the other lymphoid organs (Benner, Meima, van der Meulen and van Ewijk, 1974c).

Evidence was presented that cells producing anti-SRBC antibodies can be generated within the bone marrow itself (Benner *et al.*, 1974d). To initiate such a bone marrow PFC response to SRBC, the presence of specific memory cells seems to be required (Benner, Meima and van der Meulen, 1974a; Benner and van Oudenaren, 1975). The presence of the spleen was found to be essential for these memory cells to appear after intravenous (i.v.) priming with moderate doses of SRBC (Benner and van Oudenaren, 1975).

From the literature it is known that the thymus-independent antigen *Brucella* (Thorbecke, Asofsky Hochwald and Siskind, 1961), in contrast to thymus-dependent antigens (Thorbecke *et al.*, 1961; Langevoort, Asofsky, Jacobson, de Vries and Thorbecke, 1963), can evoke antibody formation in rabbit bone marrow during the primary response. In view of these data it seemed worthwhile to see whether mouse bone marrow can also show PFC activity after primary immunization with a thymus-independent antigen. The antigen chosen was lipopolysaccharide (LPS) from *Escherichia coli* because antibody formation to this antigen does not require helper T cells (Andersson and Blomgren, 1971; Möller and Michael, 1971) and no evidence has been presented so far that suppressor T cells play a regulatory role in the antibody response to LPS. We found that mouse bone marrow is able to show a distinct anti-LP SPFC activity, even during the primary response.

MATERIALS AND METHODS

Mice

(C57Bl/Rij × CBA/Rij)F1 female mice, 16–20 weeks old were used. They were purchased from the Medical Biological Laboratory, Rijswijk, The Netherlands, and the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

Antigen and immunization

LPS from *E. coli* O55: B5, prepared according the phenol extraction method (Westphal, Lüderitz and Bister, 1952), was obtained from Difco Laboratories, Detroit, Michigan. Before use it was dissolved in a balanced salt solution (BSS), prepared according to Mishell and Dutton (1967).

Mice were immunized by i.v. injection of doses of LPS ranging from 0·001 to 100 μg in 0·5 ml BSS. For secondary immunization mice received an i.v. booster injection 3 months after the first injection.

Splenectomy

Splenectomy and sham-splenectomy were performed 1 month before injection of LPS. Mice were anaesthetized by an intraperitoneal (i.p.) injection of 70 mg/kg body weight Nembutal (Abbott S.A., Saint-Rémy-sur-Avre, France). The incision was made in the left upper abdomen. For splenectomy the splenic blood vessels were tied in a single suture, then cut and the spleen removed. The incision was closed in two layers. There was no post-operative mortality.

Preparation of cell suspensions

Cell suspensions were prepared in BSS as described previously (Benner *et al.*, 1974d). Blood was obtained by cardiac puncture and immediately heparinized (5 u/ml). Thymi were collected 30 min after an i.p. injection with 0·1 ml of a 1 per cent solution of carbochrome ink (Gurr Ltd, High Wycombe, Buckinghamshire) in BSS. This facilitated differentiation between thymus and adherent lymph nodes in order to avoid contamination of the thymocyte suspension.

Isolation of nucleated cells from peripheral blood

Nucleated cells were isolated from mouse blood using a modification of the Ficoll–Isopaque system described by Böyum (1968). The Ficoll–Isopaque mixture was obtained by mixing 2 parts Isopaque (Nyegaard & Company, Oslo, Norway), 3 parts Ficoll (Pharmacia, Uppsala, Sweden) 9 per cent and 2 parts of a 3·5 per cent solution of bovine serum albumin in distilled water. Final density was 1·115 g/ml. Using this method at least 70 per cent of the nucleated cells was recovered. A Coulter counter model B was used for counting nucleated cells isolated from peripheral blood.

Cell counts

Viable nucleated cells were counted in a haemocytometer using 0·2 per cent trypan blue in BSS as a diluent.

Irradiation

The recipient mice received 850 rad whole body irradiation generated in a Philips Müller MG 300 X-ray machine. Animals were irradiated in well-aerated circular Perspex cages. Physical constants of

the irradiation were described previously (Benner *et al.*, 1974a). Irradiated control mice died in 9–16 days.

Cell transfer

Recipients were injected i.v. with the appropriate cell suspension and 5 μg LPS within 4 h after irradiation. On the 4th day all mice were boosted with another 5 μg of LPS i.p. PFC in the spleen of recipient mice were determined 7 days after cell transfer, since at that moment PFC activity in the recipient spleen is maximal. Each group consisted of five mice.

Assay for PFC

PFC were determined according to the method of Cunningham and Szenberg (1968) with some modifications as described previously (Benner *et al.*, 1974d; Benner and van Oudenaren, 1975). SRBC coated with LPS were used as targets. Before use LPS (1 mg/ml phosphate-buffered saline (PBS), pH 8·0) was boiled for 2 h at 120°. Coating was performed by incubation of 0·3 ml washed and packed SRBC with 0·7 ml of the heat-treated LPS solution at 37° for 45 min. The coated cells were washed three times in PBS (pH 7·2) before use in the PFC assay. The guinea-pig complement (Flow Laboratories, Rockville, Maryland) used in the plaque assay was previously absorbed with SRBC, mouse spleen cells and agarose. Absorption with agarose (3 mg/ml guinea-pig serum) was found to give excellent removal of anti-LPS antibodies (Zaalberg, personal communication). IgG and IgA producing anti-LPS PFC could not be detected at any time after primary and secondary immunization with LPS.

Calculation of total bone marrow PFC activity

Bone marrow PFC activity was determined in the femoral marrow. From the results of the femoral bone marrow the number of PFC present in the marrow of the whole animal was estimated using the data of Chervenick and coworkers (1968), who showed that in mice one femur contains 5·9 per cent of the total marrow. Comparison of the PFC responses evoked by fixed numbers of nucleated bone marrow cells from femur, tibia, humerus, rib and sternum (Table 1) indicated that anti-LPS PFC activity in various compartments of mouse bone marrow is about the same.

Passive haemagglutination titres

Anti-LPS antibody titres were assayed by means of a passive haemagglutination technique using a 1 per cent suspension of SRBC coated with LPS. The samples were tested in duplicate with the standard twofold dilution method for the presence of 2-mercaptoethanol (2-ME)-sensitive and 2-ME-resistant antibodies. For determination of 2-ME-resistant antibody titres, the sera were diluted v/v with 0·2 mol 2-ME. After incubation for 2 h at room temperature, the sera were titrated according to the standard method.

Table 1. Comparison of PFC activity in various compartments of mouse bone marrow during the primary response to LPS

Source of bone marrow cells	PFC/10^7 viable nucleated cells*
Femur	2492† (1419–4376)
Tibia	3415 (1886–6181)
Humerus	2937 (1908–4521)
Rib‡	2986 (1644–5425)
Sternum‡	2960 (1746–5019)

* Mice were immunized with 10 μg LPS i.v. PFC assay was done 5 days after immunization.

† Geometric mean and 95 per cent confidence limits.

‡ Marrow cells from ribs and sternum were obtained by gently breaking the bones with mortar and pestle. Cells were separated from bone fragments by squeezing the suspension through a nylon-gauze filter with 30-μm openings.

RESULTS

Primary response to LPS

Immunization of mice with 0·001, 0·01, 0·1, 1 and 10 μg LPS i.v. evoked a PFC response in the spleen (Figs 1 and 2). Independent of the antigen dose, peak PFC activity was always found on the 4th day after immunization. After LPS doses of 1 and 10 μg, but not after lower doses, PFC could also be detected in the bone marrow (Fig. 1). This PFC activity in the bone marrow equalled or surpassed the PFC activity in the spleen at 5 days after immunization. In all other lymphoid organs tested no significant PFC activity was found at any time after a single dose of LPS.

The absence of PFC activity in the bone marrow

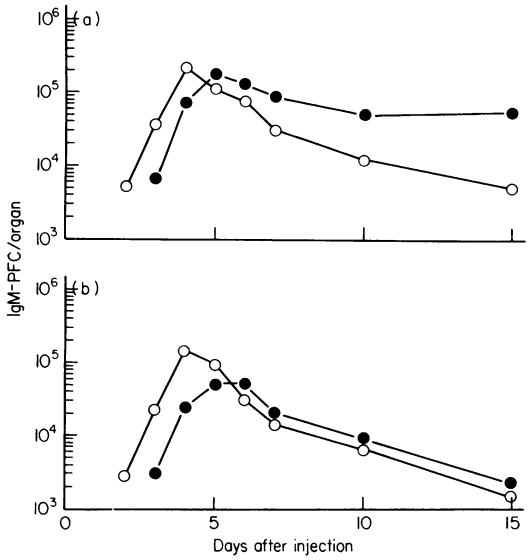

Figure 1. Number of PFC in mouse spleen (○) and bone marrow (●) after one injection of (a) 10 or (b) 1 μg LPS i.v. In lymph nodes, Peyer's patches, thymus and blood no significant PFC activity was found at any time after immunization. PFC assay was done on pooled spleens. Each group consisted of at least four mice.

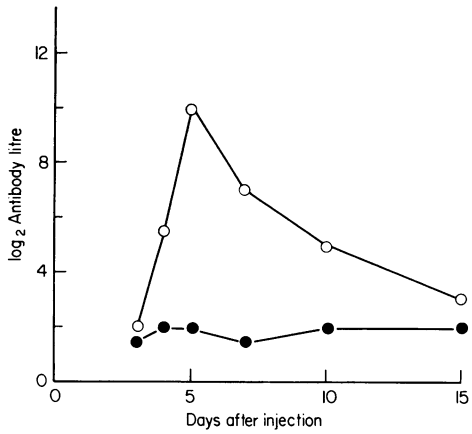

Figure 3. Agglutinating antibody titres in the sera of mice sham-splenectomized (○) or splenectomized (●) 1 month before immunization with 0·1 μg LPS i.v. The anti-LPS antibodies were 2-ME-sensitive.

after 0·1 μg or less LPS might be due to the elimination of antigen by the spleen. Therefore we also studied the response to 0·1 μg LPS in splenectomized mice. Such mice also showed no significant PFC activity in the bone marrow and no increase in the serum agglutination titre (Fig. 3). After i.v. immunization with doses of LPS as low as 0·1 μg the spleen seems to be the only site of antibody formation.

Secondary response to LPS

The secondary response to LPS was studied in mice primed with 1 μg LPS i.v. and boosted with either 10, 0·1 or 0·001 μg LPS i.v. 3 months later. After each dose tested the PFC activity in the spleen (Fig. 4) was much higher than during the primary response

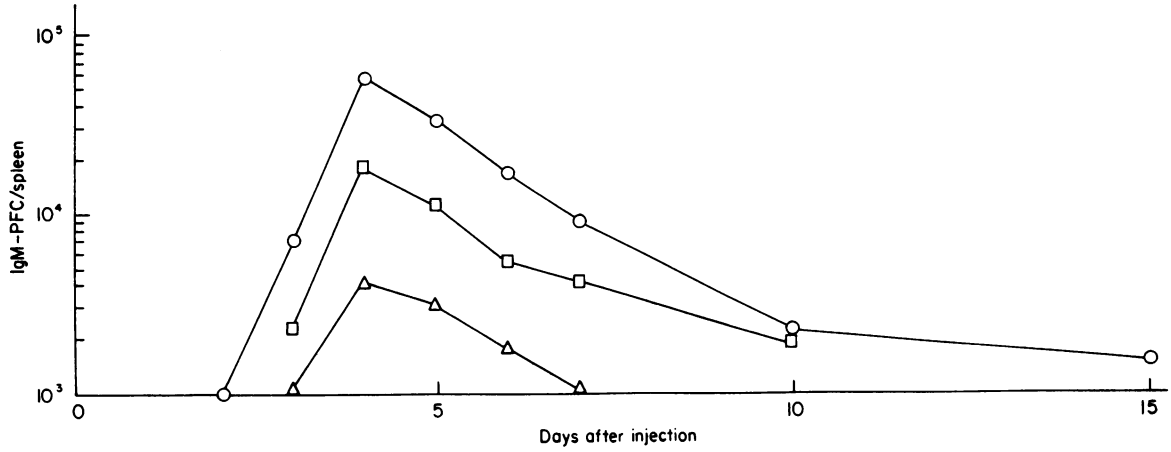

Figure 2. Number of PFC in mouse spleen after one injection of either 0·1 (○), 0·01 (□) or 0·001 (△) μg LPS i.v. In bone marrow, lymph nodes, Peyer's patches, thymus and blood no significant PFC activity was found at any time after immunization. PFC assay was done on pooled spleens. Each group consisted of at least four mice.

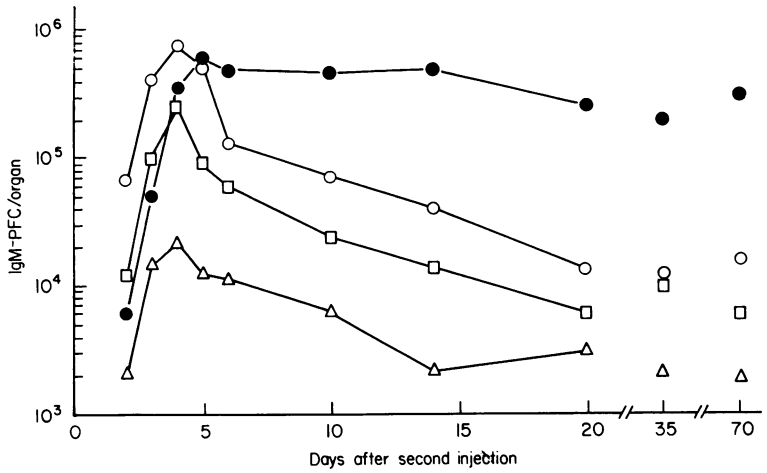

Figure 4. Number of PFC in mouse spleen and bone marrow after two injections of LPS. Mice were primed with 1 μg LPS i.v. and boosted with either 10 (○, spleen; ●, bone marrow), 0·1 (□, spleen) or 0·001 (△, spleen) μg LPS i.v. 3 months later. After boosting with 10 μg LPS i.v. PFC activity was found not only in spleen and bone marrow, but also in mesenteric lymph node, peripheral lymph nodes, Peyer's patches, thymus and blood (see Fig. 5). After boosting with 0·1 and 0·001 μg LPS i.v. no significant PFC activity was found in other lymphoid organs than spleen.

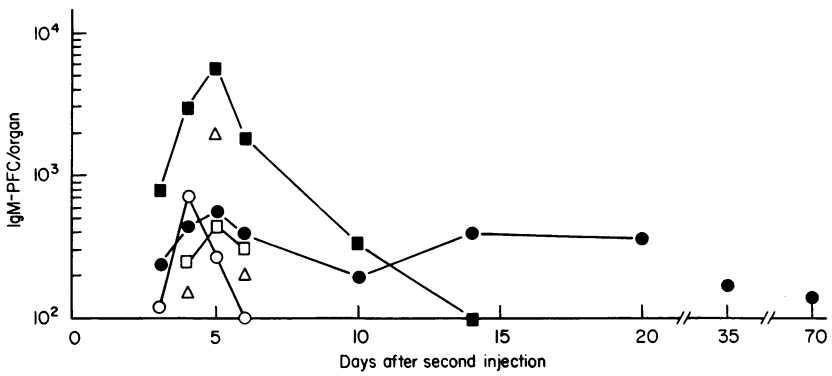

Figure 5. Number of PFC in mouse mesenteric lymph node (■), peripheral lymph nodes (○), Peyer's patches (□), thymus (●) and blood (△) after two injections of LPS. Mice were primed with 1 μg LPS i.v. and boosted with 10 μg LPS i.v. 3 months later. The organs were obtained from the same mice used to determine PFC activity in spleen and bone marrow (Fig. 4).

to that dose (compare Fig. 1, 2 and 4). Similar to the primary response after doses of 0·1 and 0·001 μg LPS i.v., these doses did not evoke a PFC response in the bone marrow. After boosting with 10 μg LPS i.v. a PFC response was found not only in spleen, but also in bone marrow (Fig. 4), mesenteric lymph node, peripheral lymph nodes, Peyer's patches, thymus and blood (Fig. 5). At about 5 days after this booster injection the number of PFC in the bone marrow exceeded the total number of PFC in all the other lymphoid organs. Although only a few PFC were found in the thymus, the response in this organ was more sustained than in lymph nodes, Peyer's patches and blood.

The influence of the priming dose of LPS on the appearance of B memory cells was studied by means of cell transfer experiments. Seven days after transfer of 2×10^7 viable nucleated non-immune spleen cells, bone marrow cells or thymus cells together with 5 μg LPS into lethally irradiated mice 49,500, 11,250 and

(a not significant number of) 175 PFC could be detected in the recipient spleen respectively. When the donor mice had been primed with 0·1, 1, 10 or 100 μg LPS i.v. three months before, spleen cells, bone marrow cells and thymus cells were able to evoke increased anti-LPS PFC responses in the recipient spleen. The relationship between the priming dose and adoptive PFC response was found to be an optimum curve (Fig. 6). LPS doses of 1 and 10 μg i.v. gave the best priming; 1 μg was used in the experiments on secondary responses described above.

The relatively low adoptive PFC response evoked by cells from mice primed with 100 μg LPS i.v. (Fig. 6) is temporary. This was shown by transferring

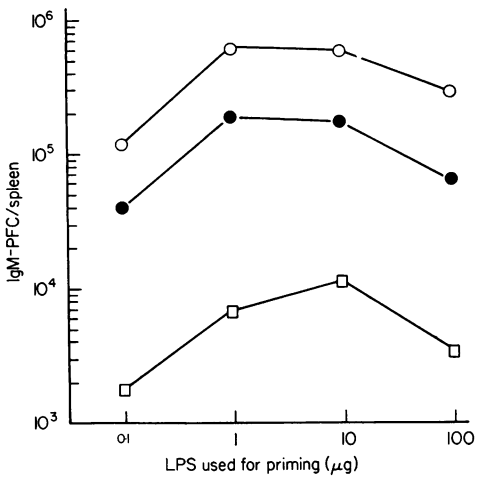

Figure 6. Adoptive PFC response of spleen, bone marrow and thymus cells 3 months after priming with various doses LPS i.v. (○) Spleen cells, (●) bone marrow cells and (□) thymus cells. Viable nucleated cells (2×10^7) were transferred to lethally irradiated syngeneic mice together with 5 μg LPS. Each group consisted of five recipient mice. PFC assay was carried out on pooled spleens 7 days after cell transfer. Transferred spleen cells, bone marrow cells and thymus cells from non-immune mice evoked 49,500, 11,250 and 175 PFC respectively.

spleen cells, bone marrow cells and thymus cells from such mice 6 months after priming. At that time adoptive PFC responses were much higher than after 3 months (Table 2). Apparently high doses of LPS i.v. result initially in a smaller number of B memory cells than do moderate doses of LPS. Hanna and Peters (1971) reported similar results for the appearance of B memory cells in the spleen after immunization with SRBC. These authors ascribe the small

Table 2. Adoptive PFC response by spleen, bone marrow and thymus cells 3 and 6 months after priming with 100 μg LPS i.v.

Cells transferred from: *	PFC/recipient spleen†	
	3 months	6 months
Spleen	272,900	872,500
Bone marrow	58,100	179,250
Thymus	3200	8025

* Viable nucleated cells (2×10^7) and 5 μg LPS were transferred to lethally irradiated syngeneic mice.

† PFC assay was done on pooled spleens 7 days after cell transfer. Each group consisted of five recipient mice. Figures from a representative experiment.

numbers of B memory cells after high doses of antigen to an exhaustion of this cell population, due to maturation of newly formed B memory cells into antibody-forming cells.

DISCUSSION

After primary immunization of rabbits with the thymus-independent antigen *Brucella*, bone marrow cells of these animals cultured *in vitro* were found to release specific antibodies (Thorbecke *et al.*, 1961). As far as antibody formation *in vivo* to thymus-independent antigens has been studied by means of the plaque assay, most frequently PFC activity has been determined in spleen and lymph nodes. Only a few authors have studied the bone marrow as a source of PFC (Landy, Sanderson and Jackson, 1965; Baker, Stashak, Amsbaugh and Prescott, 1971). Landy *et al.* (1965) found small but significant numbers of PFC in the bone marrow of rabbits after primary immunization with *Salmonella enteritidis* polysaccharide. On the other hand Baker *et al.* (1971) could not detect any PFC activity in mouse bone marrow during the primary response to an optimal dose of type III pneumococcal polysaccharide (SIII).

The results presented in this paper show that mouse bone marrow can exhibit a clear PFC activity to the thymus-independent antigen *E. coli* LPS, both during the primary (Fig. 1) and the secondary response (Fig. 4). In previous papers of this series

(Benner *et al.*, 1974c, d, 1975) it has been shown that mouse bone marrow can contain large numbers of PFC to the thymus-dependent SRBC antigens, however, only during the secondary, and not during the primary response. Specific memory cells are probably required for the appearance of anti-SRBC PFC in the bone marrow during the secondary response (Benner *et al.*, 1974a, 1975). Splenectomy experiments (Benner *et al.*, 1974d; Benner and van Oudenaren, to be published) provided evidence for the *in situ* differentiation of non-antibody-producing cells into anti-SRBC and anti-LPS antibody-forming cells in mouse bone marrow. The most tempting explanation why anti-SRBC PFC activity in the bone marrow is a memory-dependent phenomenon, in contrast to anti-LPS bone marrow PFC activity, is related to the difference in thymic dependency between these two antigens. While after immunization with LPS B cells which are normally present in mouse bone marrow might differentiate without T cell help into PFC *in situ*, SRBC-specific B cells are unable to do so because they need the presence of T memory cells.

It is notable that anti-LPS PFC responses in the bone marrow appear only after high doses of antigen. This observation and the absence of PFC activity in the bone marrow after i.p. immunization with an optimal dose of SIII (Baker *et al.*, 1971) suggest that antibody formation to these thymus-independent antigens in the bone marrow is not only dependent on the presence of the appropriate B cells. *In vitro* experiments suggest that macrophages are involved in antibody formation to LPS (Bona, Robineaux, Anteunis, Heuclin and Astesano, 1973) and SIII (Aaskov and Halliday, 1971). The appearance of anti-LPS PFC in the bone marrow after immunization with 1 or 10 μg LPS (Figs 1 and 4) might therefore be due to interference by LPS with the reticuloendothelial system (RES). Benacerraf and Sebestyen (1957) showed that doses of 10 μg LPS i.v. cause a transitory depression of the phagocytic activity of the RES. Within one day this depression is followed by a period of increased phagocytic activity. When the presentation of antigen to lymphocytes in the bone marrow is the limiting factor for initiation of an anti-LPS PFC response *in situ*, stimulation of the phagocytic activity of the RES probably accounts for the appearance of PFC in the bone marrow after immunization with relatively high doses of LPS.

It is of interest to compare the minimal threshold antigen dose of LPS and SRBC in spleen and bone marrow. The threshold dose of LPS for the bone marrow was about a thousand times higher than for the spleen. Such a great difference in the threshold antigen dose for spleen and bone marrow was not apparent during the secondary response to SRBC (Benner *et al.*, 1974c). Several causes may account for this difference. In our opinion the most probable ones are related to the presentation of the antigen to lymphocytes in these organs with an anatomically so different structure.

(1) In contrast to SRBC, LPS is a polymeric antigen and is composed of much smaller particles (Shands, 1971). Therefore red blood cells might be more susceptible to antigen-processing mechanisms in the bone marrow than LPS.

(2) LPS only evokes the production of IgM antibodies; SRBC, however, can also evoke the formation of IgG antibodies which have a much longer half-life and are better opsonins than IgM (Spiegelberg, 1974). At the moment of the second injection of SRBC opsonic antibodies from the primary response cause an optimal antigen-processing in the bone marrow. On the other hand, when mice receive a primary injection of LPS or a second one 3 months later, little or no specific IgM is present. Consequently antigen-processing within the bone marrow could be deficient.

In some respects there is a striking similarity between the shape of the spleen and bone marrow PFC curves during the secondary response to SRBC (Benner *et al.*, 1974c, d, 1975) and the response to 10 μg LPS. For both antigens the bone marrow PFC response is characterized by: (1) a delayed appearance of PFC as compared with the spleen; (2) a much higher PFC activity than in all other lymphoid organs together during the second phase of the response. The underlying regulating mechanisms of these phenomena are unclear. The delay of appearance of PFC in the bone marrow might be related to differences in antigen processing between spleen and bone marrow. Alternatively this delayed appearance of PFC might be due to a time-consuming migration of cells involved in the bone marrow PFC response into this organ during the first phase of the response. Although in previous papers (Benner *et al.*, 1974d, 1975) migration of PFC from peripheral lymphoid organs into the bone marrow could be excluded as the underlying cause for PFC activity in the bone marrow, the possibility of immigration in the bone marrow of cells triggered to give a PFC response has to be considered. The high bone marrow PFC

activity during the second phase of the response is possibly due to one or more local factors which enhance the differentiation into PFC within the bone marrow. Recently it was reported that bone marrow cells can synthesize a humoral factor which enhances the PFC response in cultures of lymph node cells obtained from immune donors at the peak of the productive phase of antibody formation (Petrov, Mikhajlova, Stepanenko and Zakharova, 1975). This factor might account for the high PFC activity observed in the bone marrow during the second phase of the response.

The appearance of B memory cells in the thymus after i.v. immunization with LPS (Fig. 6) is consistent with previous experiments with SRBC as antigen (Jehn and Karlin, 1971; Benner, Meima and van der Meulen, 1974b). The presence of B memory cells in the thymus coincides with the capacity of this organ to exhibit PFC activity upon subsequent stimulation with the antigen used for priming. This suggests that the appearance of PFC in the thymus during the secondary response is due to an *in situ* differentiation of B memory cells into PFC. Thymic PFC responses can only be evoked by high doses of antigen (Fig 5) (Benner *et al.*, 1974c), probably because antigens scarcely enter the thymus tissue (Kater, 1970).

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