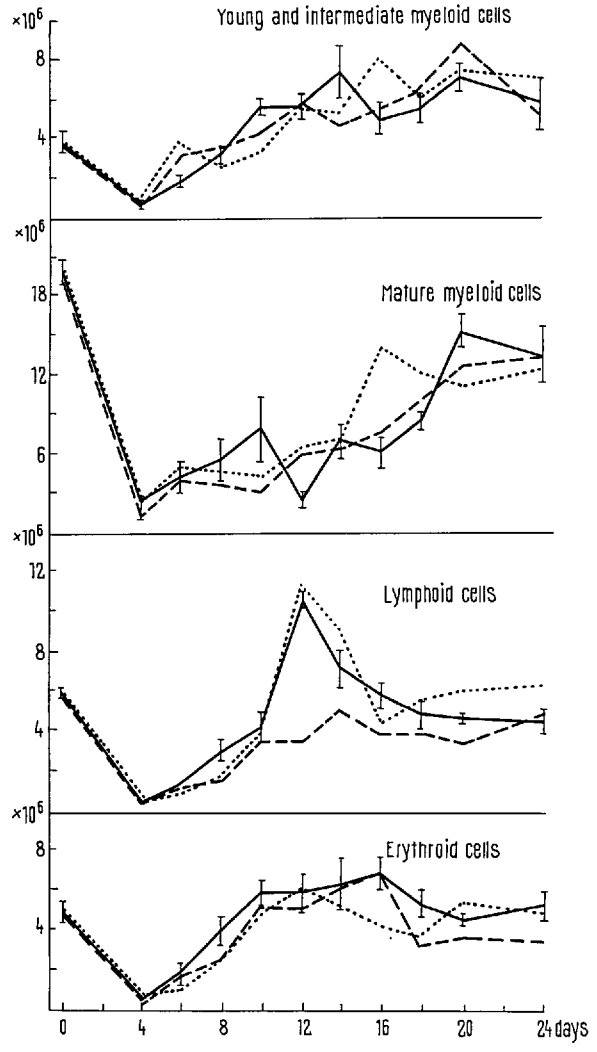

Fig. 2. Absolute numbers of various cell types after 500 R (——), 500 R with a normal marrow graft (– – –), and 500 R with an irradiated marrow graft (···).

marrow and is able to inhibit the development of the lymphoid population. This factor should be destroyed by the irradiation with a subsequent release of the lymphoid growth. A normal marrow graft would provide a sufficient amount of the inhibitory factor to restore its effect.

An alternative possibility is a competition between normal and irradiated marrows. The peculiar lymphoid (X cell) population, which constitutes an abnormal cell line in the adult marrow[4], should be overgrown by the normal corresponding cell line derived from the graft, i.e. from normal marrow which has been shown to recover without any lymphoid rebound[6].

However, the mechanisms regulating these cell populations are till now unknown. Thus other hypotheses can certainly not be discarded, even those involving extramedullary feed-back controls which could be perturbed by the irradiation and restored by a normal marrow graft.

Résumé. Après une irradiation sublétale, la mœlle présente une accumulation transitoire de cellules lymphoïdes particulières (cellules transitionnelles ou cellules X). Cette accumulation n'apparaît pas après une greffe de mœlle normale. Le comportement des cellules lymphoïdes n'est pas influencé par une greffe de mœlle irradiée.

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'High Dose' Immunologic Tolerance to *Escherichia coli* Lipopolysaccharide Assessed by Bacteriolytic and Hemolytic Plaque Assays

Immunologic tolerance or paralysis can be induced in experimental animals with highly immunogenic atigens derived from Gram-negative bacteria. Examples include the immunologic unresponsiveness induced in neonatal rodents to lipopolysaccharide (LPS) antigens of *Shigella paradysenteriae* and *Escherichia coli*, as well as to purified protein antigens derived from *Salmonella flagellin*[1–13]. Cellular aspects of tolerance to the LPS antigens have been investigated by means of a modified Jerne hemolytic plaque assay in agar gel, using target sheep erythrocytes coated with the appropriate bacterial extract[3,4,9–11]. Immobilization tests and bacterial adherence have been used to assess cellular events during tolerance to the flagellar antigens[6,12,13].

In studies with the *E. coli* LPS, tolerance has been achieved in both neonatal and adult mice[9–11,14–16]. Mice injected repeatedly as adults with the *E. coli* antigen showed a marked depression in their ability to respond to challenge immunization with this antigen, but not others[10,11]. However, experiments using both the passive hemolytic plaque assay, with antigen-sensitized sheep red blood cells, and a more direct bacteriolytic assay with viable bacteria as the indicator, resulted in contrasting results. A much greater suppression was evident when the passive hemolytic plaque assay was used to enumerate antibody forming cells[14–16]. In contrast, many more antibody-producing cells were found in the same spleen cell suspensions when the direct bacterial plaque procedure, with viable bacteria, was used as the indicator. Such findings were consistent with the concept that tolerance to antigens as complex as the somatic extracts of bacteria is most likely due to unresponsiveness to 'major' antigens present in the extract which may not sensitize the indicator erythrocytes[16].

In the earlier studies of BRITTON[10,11], tolerance was induced with relatively large concentrations of a detoxi-

fied LPS extracted from *E. coli*. The number of cells producing antibody in test lymphoid cell suspensions was determined only by the passive hemolytic plaque assay with LPS-coated red blood cells. Challenge immunization consisted of injection of a whole cell *E. coli* vaccine. Use of the passive plaque assay could have obscured a demonstrable immune response to some of the other antigens of this microorganism, since any antibody forming cell with specificity to antigenic determinants besides those coating onto the red blood cells would be undetected.

In the present study mice were injected as adults with a relatively large concentration of a detoxified LPS extract from *E. coli* and then tested for development of 'high dose' tolerance, using both the passive hemolytic plaque assay and the direct bacteriolytic immunoplaque procedure. The number of specific antibody plaque forming cells (PFC) was assessed after challenge immunization with the *E. coli* vaccine.

For these experiments groups of adult inbred CBA mice, each weighing approximately 18–24 g, were injected i.v. into the tail vein with 10 mg *E. coli* 0127:B8 LPS (Difco Laboratories, Detroit, Mich.), prepared by the phenol extraction procedure and detoxified exactly as described by NETER et al.[17]. Each of the mice was injected either once with the entire dose of antigen or 5 times over an 8 day period with 2.0 mg doses (Table). 8 days later all mice were challenged by i.v. injection of approximately 15×10^7 heat killed *E. coli*. 6 days later the mice were killed and the total number of PFC per spleen determined by the 2 assays, exactly as described elsewhere[18,19]. Serum agglutinin titers were also determined, as described elsewhere[19]. For the plaque assay, the mean number of hemolytic (H-PFC) and bacteriolytic (B-PFC) immunocytes was calculated for each group.

As can be seen from the Table, injection of 10 mg of the detoxified LPS, either as a single injection or in repeated doses, resulted in appearance of approximately 20,000–100,000 antibody forming cells in the spleens of the test animals after challenge, as determined by the bacteriolytic assay. When the same spleen cell suspensions were assessed by the passive hemolytic procedure, using the LPS sensitized erythrocytes, the number of H-PFCs was considerably lower (generally in the range of 3000 per spleen). In contrast, when control non-treated mice were challenged with the same LPS antigen, there was a rapid appearance of both B-PFC and H-PFC. As can be seen from the Table, about 115,000 plaque forming cells were detected by the bacteriolytic procedure, and approximately half that number by the hemolytic assay.

It should be noted that the numbers of PFCs, both bacteriolytic and hemolytic, were *higher* in non-challenged, LPS-treated mice (Group E) tested at the same time interval as the LPS-treated mice (Group A) which were challenged with the whole cell vaccine 6 days before assay. If this number of PFCs was subtracted from the response of the other treated mice, the tolerance assessed by the H-PFC method would be complete. Similar observations were made by BRITTON[10,11].

Agglutinin titers to the whole bacteria were only slightly different in sera of mice given either the single or repeated large doses of antigen before challenge immunization (Table). Both control and antigen treated animals had agglutinin titers averaging 1:64–1:128 after challenge. The hemagglutinin and hemolysin titers with LPS sensitized erythrocytes were somewhat lower (Table).

The number of 'background' PFC was generally similar with either sensitized erythrocytes, untreated RBCs, or viable bacteria. There were generally fewer than 60 background PFCs per spleen in non-treated mice with all indicator antigens (Group D). Injection of the large

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Appearance of antibody-forming cells to *E. coli* in spleens of adult CBA mice given a large dose of LPS before challenge immunization with *E. coli* vaccine

Mouse group[a]	Individual antigen dose injected (mg)	Total antigen dose (mg)	Challenge immunization[b]	Antibody plaque forming cells per spleen			Agglutinin titer (log_2)	Hemagglutinin titer (log_2)	Hemolysin titer (log_2)
				Bacteriolytic	Passive hemolytic	Hemolytic (control RBC)			
A	10.0	10.0	+	19,440	3,350	160	5.8	3.0	4.1
B	2.0	10.0	+	97,282	3,033	254	6.8	2.6	4.0
C	0	0	+	115,444	62,646	99	6.8	3.1	4.7
D	0	0	—	60	24	45	<3	1.7	2.7
E[c]	10.0	10.0	—	35,463	7,610	84	6.7	2.7	3.6

[a] Groups of 6–15 mice injected i.v. as indicated with detoxified LPS. [b] Mice challenged by i.p. injection of 15×10^7 heat killed bacteria 8 days after initial injection of LPS; antibody response determined 6 days later. [c] Response of non-challenged LPS-injected mice tested for PFC at same time as challenge mice.

amount of detoxified LPS stimulated a sharp increase in the number of PFCs to untreated RBCs; this increase was usually greater than that observed after challenge immunization of the normal mice (Table 1, Group C).

The results of this study confirm and extend the experiments of BRITTON concerning induction of immunologic paralysis in mice with a relatively large concentration of detoxified LPS[9–11]. However, in that study only hemolytic plaques were assayed using LPS-sensitized sheep RBCs. Such an assay indicated the development of a profound immunosuppression or tolerance, since very few hemolytic PFCs to *E. coli* LPS appeared after challenge with the whole cell vaccine. However, it might be expected that such unresponsiveness would be directed only towards the LPS, and not to other antigenic determinants which might be present on the intact bacterium. Thus it was somewhat unexpected in this study that treatment of mice with 10 mg detoxified LPS also reduced the number of B-PFCs, as well as H-PFCs, in comparison to the responses of control mice. This reduction was most evident in the mice given a single large dose of LPS. Mice given smaller doses, in 5 injections, had approximately a 20% reduction in the number of B-PFCs and nearly 95% reduction in the number of H-PFCs.

These findings support the concept that the mice were tolerant mainly to the major LPS determinant and not to other 'minor' determinants which may be present in the LPS but not attached to the indicator sheep red cells[15,16]. However, it is noteworthy that mice injected once with the 10 mg dose of antigen showed a 70–80% further reduction in the number of B-PFCs. Thus, either specific tolerance to the whole bacteria was induced in these animals or a 'non-specific' immunosuppression developed due to injection of this relatively large quantity of somatic antigen. The later possibility seems unlikely since BRITTON, in similar experiments, found that the induction of tolerance was specific as judged by the ability of tolerant mice to respond normally to sheep erythrocytes[10,11].

It also seems unlikely that the immunosuppression observed in this study was due merely to 'feed back' suppression by antibody. In other experiments it was found that little if any serum antibody could be detected before challenge[15]. Furthermore, lower doses of this LPS, which stimulate little antibody formation, could induce similar tolerance when injected repeatedly during the first 6 weeks of life[15]. Thus it appears that tolerance to the LPS antigen in mice, especially that induced by a large concentration of detoxified material, may result in a marked diminution in the number of antibody producing cells with specificity towards the intact bacteria, as well as to the purified LPS[20].

Zusammenfassung. Immunologische Toleranz konnte in Mäusen durch hohe Dosen von *E. coli* Lipopolysaccharid erreicht werden. Der Effekt war mit Hilfe der passiven hämolytischen «plaque»-Methode besser zu erfassen als im direkten bakteriologischen System.

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Bacterial Endotoxins as Immunosuppressive Agents

Endotoxins from gram-negative bacteria may act as adjuvants in the primary immune response[1]. Studies at the cellular level by means of the direct antibody plaque technique[2] showed that the adjuvancy of lipopolysaccharides is associated with an accelerated development of 19S hemolysin-forming cells[3–5]. Recent studies resulted in the finding that the simultaneous injection of sheep red blood cells (SRBC) and bacterial endotoxin (ET) leads to the development of enhanced peak values of 19S and 7S hemolysin-producing spleen cells, as compared to the injection of SRBC alone[6]. This effectiveness is associated with a significantly increased process of priming for the secondary response[7]. In contrast, it has been reported that the injection of relatively large amounts of a bacterial lipopolysaccharide, together with a primary antigenic stimulus, resulted in suppression of antibody formation instead of an enhanced response[8,9]. Such suppressive effects were likewise observed in mice, if considerably smaller amounts of endotoxins (10–100 μg per animal) were administered a few days before antigen[10]. On the other hand, a normal hemolysin response was found after the simultaneous injection of SRBC and ET in endotoxin-tolerant mice[4]. From this it was suggested that precursors of hemolysin-producing cells are not lost or altered in endotoxin-tolerant mice. Unfortunately, only the 2nd day after immunization was studied[4]. Therefore it appeared worthwhile to find out, at the cellular and humoral level, whether or not the primary immune response is diminished in mice pretreated with different amounts of ET.

Experiments were performed using male mice of the strain NMRI (19–26 g). In a first series of experiments 4 mouse groups were employed. The animals of group I received an i.p. injection of 4×10^8 SRBC on day 0. Mice of group II were additionally treated by an i.v.

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