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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[20] Supported in part by grants from the U.S. National Science Foundation and the National Institutes of Health, Bethesda, Maryland.

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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injection of 100 µg ET from *Serratia marcescens* (Difco, Code 3130), administered simultaneously with the SRBC. Mice of group III immunized in the same manner as those of group II were pretreated i.p. by 20 µg of the ET from *S. marcescens* daily for 9 days (day −9 to day −1). Mice of group IV serving as further controls received a single dose of 100 µg ET from *S. marcescens* only, administered i.v. at the time when the other experimental groups were immunized. In a second series of experiments, the mice were primarily immunized by an i.p. injection of 4×10^8 SRBC (group I) or 4×10^8 SRBC and 100 µg ET from *S. mercescens* (groups II to IV). For pretreatment either ET from *S. marcescens* (group III) or ET from *S. typhi* (Difco, Code 3124) (group IV) was used. Designating the day of immunization as day 0, 25 µg of the ET preparations were given i.p. on days −9, −8 and −7, 50 µg on days −6, −5 and −4, 100 µg on days −3, −2 and −1, respectively.

At different intervals after the immunization 5–7 mice out of each group and 2 animals of the untreated controls were sacrificed, the spleens removed aseptically, the sera collected and after pooling stored at −20 °C until use. For the quantitative determination of 19S and 7S hemolysin-producing spleen cells, the direct[2] and indirect[11] antibody plaque techniques were used, as described elsewhere[6,7]. It is generally accepted that the direct plaque-forming spleen cells (PFC) represent 19S producers, whereas the majority of the indirect PFC are considered as 7S producers[11]. Total serum hemolysin activity of pooled serum samples and of fractions resistant to treatment with 0.125 *M* 2-mercaptoethanol(2-ME) were determined spectrophotometrically at 530 nm by the 50% hemolysis method. Total and 2-ME-resistant agglutinin titers of pooled sera were assayed by a standard tube method.

As compared to the primary immunization of mice with 4×10^8 SRBC (group I), the additional injection of 100 µg ET from *S. marcescens* (group II) led to an accelerated and prolonged development of direct PFC and indirect PFC. In both groups of mice the mean peak values of direct PFC were found on day 4 (Figure 1). The differences of the mean values of direct PFC were statistically significant on day 5 ($t = 2.9044$; $2P < 0.02$) and on day 5.5 ($t = 2.2289$; $2P < 0.05$). For the group I the maximal peak values of indirect PFC were determined 4.5 days after the primary immunization, followed by a sharp drop. A second peak was found on day 10 (Figure 1). Opposite to this, the average peak value of indirect PFC in the spleens of group II was found on day 5, differing

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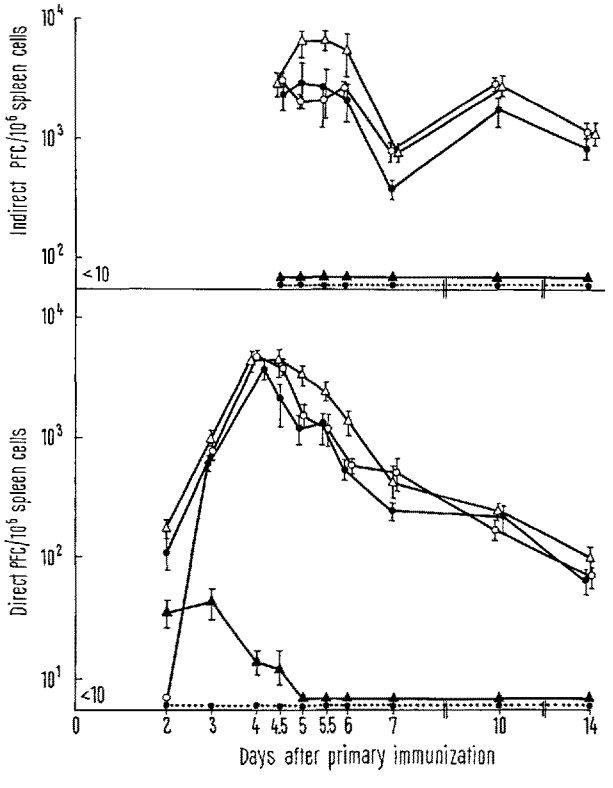

Fig. 1. Development of direct and indirect plaque-forming spleen cells (PFC) in mice after the primary immunization with 4×10^8 sheep red blood cells (SRBC) and 100 µg endotoxin (ET) from *S. marcescens* in normal mice (△, group II) or animals pretreated with 20 µg of the same ET daily for 9 days (●, group III), as compared to that of normal mice either after the injection of 4×10^8 SRBC (○, group I) or the injection of 100 µg ET from *S. marcescens* (▲, group IV). (...), untreated controls.

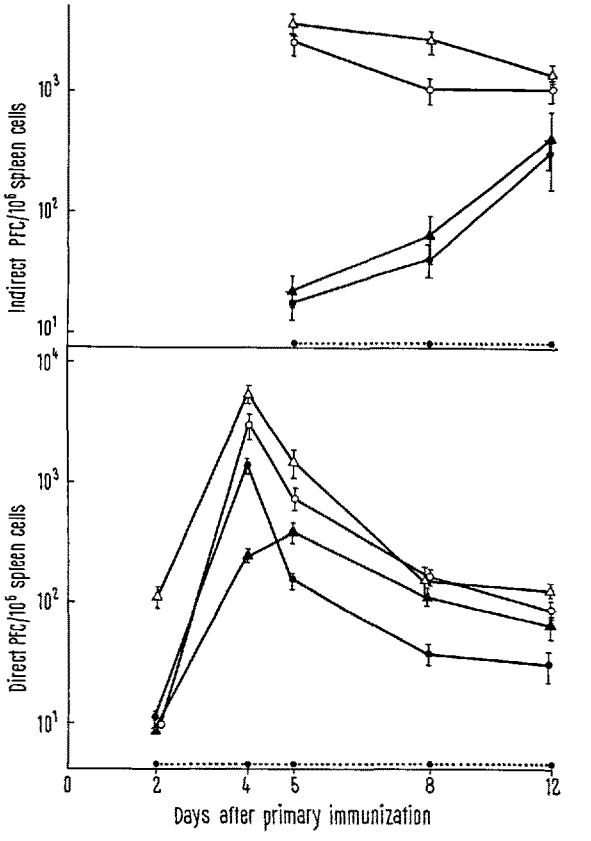

Fig. 2. Development of direct and indirect plaque-forming spleen cells (PFC) in mice after the primary immunization with 4×10^8 sheep red blood cells (SRBC) and 100 µg endotoxin (ET) from *S. marcescens* in normal mice (△, group II) or animals pretreated with relatively large amounts of ET from either *S. marcescens* (●, group III) or *S. typhi* (▲, group IV), as compared to that of normal mice after the injection of 4×10^8 SRBC (○, group I). (···), untreated controls.

significantly from that of group I ($t = 2.6227$; $2P < 0.025$). Significant differences were also demonstrable on day 5.5 ($t = 3.0818$; $2P < 0.02$) and on day 6 ($t = 3.0066$; $2P < 0.02$) (Figure 1). No adjuvant activity was demonstrable when the simultaneous injection of SRBC and ET was given mice pretreated by 20 µg *S. marcescens* endotoxin daily for 9 days (group III). In the spleens of mice treated alone by an intravenous injection of 100 µg ET from *S. marcescens* (group IV) only a short-term multiplication of the relative numbers of direct PFC was detectable (Figure 1). The adjuvant effectiveness of ET from *S. marcescens* was observed again in the second series of experiments (Figure 2). But the development of both direct and indirect PFC was found to be significantly suppressed when 4×10^8 SRBC and 100 µg ET from *S. marcescens* were simultaneously injected into mice pretreated with relatively large amounts of ET from either *S. marcescens* (group III) or *S. typhi* (group IV) (Figure 2). Serological investigations resulted in similar findings.

The data presented here give evidence that pretreatment of mice with a constant dose of 20 µg ET per day during a period of 9 days inhibits the adjuvant activity of ET, whereas the normal hemolysin response was not found to be altered. A significantly diminished primary immune response was found, however, when pretreatment was performed with larger amounts of ET (Figure 2). In general it is accepted that the uptake of particulate antigens by macrophages is a necessary prerequisite for the initiation of the primary immune response[12]. Lipopolysaccharides persist for a long period in the body and may cause toxic damage of cells in blood and tissues[1]. Thus it could be suggested that the repeated administration of relatively large amounts of ET effects an exhaustion of the reticulo-endothelial system (RES) meaning that the immunosuppressive effect induced by ET would be mainly due to an injury of the afferent limb of the immune apparatus. This concept is supported by the observation that RES 'blockade' induced by injection of carbon effects a significant suppression of the development of hemolysin-producing spleen cells[12]. At first sight it appears to be less probable that the immunosuppressive effect of ET is mainly due to a loss of progenitor immunocytes. But it may be recalled that there are some signs for the pluripotential character of progenitor immunocytes, thus possibly being able to differentiate along some path other than the immunological one[13,14]. Taking such considerations into account, it is conceivable that the repeated injection of increasing amounts of bacterial lipopolysaccharides can lead to reduction in progenitor immunocytes, since the injection of ET is regularly followed by an enormous increase in white blood cells[1,6].

Zusammenfassung. Der Adjuvanseffekt von bakteriellem Endotoxin gegenüber Schaferythrocyten war nicht nachweisbar, wenn Mäuse vor der primären antigenen Stimulierung 9 Tage lang eine tägliche Injektion von 20 µg Endotoxin erhielten. Andererseits wurde unter diesen Bedingungen eine Primärreaktion gefunden, wie sie nach alleiniger Injektion des Erythrocytenantigens zur Ausbildung gelangt. Die Zahlen an 19S- und 7S-Antikörper bildenden Zellen sowie die Serumantikörpertiter waren jedoch signifikant vermindert, wenn vor Applikation der immunisierenden Injektion höhere Dosen von homologem oder heterologem Endotoxin gegeben worden waren.

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Binding of Aflatoxins B_1 and G_1 to Human Serum Proteins

Recently RAO et al.[1], using fluorescence technics, reported that aflatoxin B but not G was bound with plasma albumin when crude aflatoxins were incubated with human plasma. We have used ^{14}C-labelled pure aflatoxins to re-examine this problem, and have found that aflatoxin B_1 is bound mainly with γ-globulin, whereas aflatoxin G_1 is bound mainly with albumin.

Materials and methods. Pure aflatoxins B_1 and G_1 labelled with ^{14}C, specific activity 5.8 and 2.7 µC/µmole respectively, were prepared according to ADYE and MATELES[2]. Both 'normal clinical chemistry control serum' (Hyland, Division Travenol Laboratories, Los Angeles, Calif.) and serum collected from healthy individuals were used.

Individual 0.5 ml portions of serum were incubated with the ^{14}C-toxins (23.6 µg of B_1 and 15.8 µg of G_1) for 2 h at 37 °C, and 5 µl aliquots then subjected to paper electrophoresis as previously described[1]. Another 5 µl aliquot was mixed with BRAY's solution[3] and counted in a liquid scintillation counter. The remaining material in the tube was dialyzed through cellophane against 0.01 *M* phosphate buffer (pH 7) at 4 °C for 24 h. The undialyzed material was subjected to paper electrophoresis and liquid scintillation counting as above.

For toxin detections the electrophoretic strips were placed in contact with Sakura X-ray film in cassette, and the film developed after 60 days exposure. Unstained electrophoretograms were viewed under UV and the bands compared with those seen on stained strips. The electrophoretograms were dissected into 4 portions as shown in Figure 2B. The paper sections were counted in a liquid scintillation counter according to GEIGER and WRIGHT[4].

Results and discussion. Experiments were first carried out with plasma samples exactly by the procedure of RAO et al.[1] except that pure aflatoxin B_1 or G_1 was used. Since the results were different from those of RAO et al. the procedures used by these investigators were modified by using serum samples instead of plasma, pure

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