RAPID BREAKING OF TOLERANCE AGAINST \textit{ESCHERICHIA COLI} LIPOPOLYSACCHARIDE IN VIVO AND IN VITRO

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Many features of the induction of immunological tolerance are still unclear (for review, see reference 1). The actual mechanism by which immunocompetent cells are made tolerant is unknown. Some studies suggest that tolerance means the killing of immunocompetent cells (2–4), others that paralyzed cells exist in the organism (5–8). The finding of an increased number of antigen-binding cells in animals tolerant to thymus-independent antigens, such as \textit{Escherichia coli} lipopolysaccharide (9, 10) and pneumococcal polysaccharide (11), as compared to normal, unimmunized animals may be interpreted as the presence of paralyzed cells, in this case with persisting receptors for the antigen. Antigen-binding cells also exist in animals tolerant against thymus-dependent antigens, although their number is not increased (12–14).

The present experiments were aimed at investigating whether or not it is possible to break tolerance under conditions where recruitment of new precursor cells from stem cells cannot occur. We tried to break tolerance by transfer of spleen cells into irradiated syngeneic recipients or by incubation of tolerant cells in vitro.

\textbf{Materials and Methods}

\textit{Mice.}—2–5-months old mice of the F1 hybrid (A × C57BL) were used.

\textit{Extraction and Detoxification of E. coli Lipopolysaccharide (CPS).}—Lipopolysaccharide was obtained from \textit{E. coli} 055:B5 by phenol extraction according to the method of Westphal et al. (15) with minor modifications (16). Detoxification was performed with weak alkali as described by Neter et al. (17).

\textit{Immunization.}—For an optimal antibody response 75 × 10^6 killed \textit{E. coli} 055:B5 bacteria were injected intravenously. When a spleen cell transfer was performed the bacteria were injected together with the cells. The numbers of plaque-forming cells (PFC) in the spleens were assayed 6 days after immunization if not otherwise stated.

\textit{Tolerance Induction.}—Tolerance was induced either by five injections of 3 mg of alkali-detoxified CPS each over a period of 14 days or alternatively, by two injections of 5 mg of detoxified CPS each, with a 4 day interval between injections. Tolerance was thereafter maintained by weekly injections of 3 mg of CPS each for 1–4 wk before use of the mice.

\textit{PFC Assay.}—Spleen cell suspensions were prepared by pressing the spleens through a 60 mesh steel screen into balanced salt solution (BSS). After washing the cells were suspended to the appropriate cell concentration in BSS. The number of PFC was assayed according to the method of Jerne and Nordin (18) as modified by Mishell and Dutton (19) for the use of microscope slides. Duplicate slides were made for each cell suspension. Cellular antibody

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\textit{Abbreviations used in this paper:} B cells, bursa-equivalent lymphocytes; BSS, balanced salt solution; CPS, \textit{E. coli} lipopolysaccharide; PFC, plaque-forming cells; T cells, thymus-derived lymphocytes.
synthesis to CPS was studied by coating sheep red blood cells with CPS according to Möller (20). To calculate the number of PFC against CPS, the number of PFC against untreated sheep red cells was subtracted from the number of PFC against CPS-sensitized sheep red cells obtained with the same cell suspension. Since CPS stimulates IgM antibody synthesis exclusively for a prolonged period (21), only direct PFC were studied.

**Cell Transfer.**—Spleen cell suspensions were prepared as described above. The spleen cells were spun down at 400 g once and resuspended in BSS. 10 × 10^6 spleen cells were injected intravenously together with 75 × 10^6 *E. coli* bacteria into syngeneic recipients irradiated with 600 R in a Siemens X-ray machine (Siemens Corp., Iselin, N.J.) at 15 ma and 185 kv.

**Incubation In Vitro.**—Spleen cells were cultured for 20 hr at a cell density of 10 × 10^6 spleen cells/ml according to the conditions described by Mishell and Dutton (19). Thereafter they were washed, suspended in BSS, and injected into irradiated recipients together with *E. coli* bacteria.

**RESULTS**

**Kinetics of the Normal Immune Response.**—To determine the peak of the antibody response against CPS, the number of PFC in the spleen was studied at various times after immunization. In normal mice the number of PFC was maximal on days 4 and 6 and had declined on day 8. This was true both for the number of PFC per spleen and the number per 10^6 spleen cells (Fig. 1).

If normal spleen cells were transferred into irradiated recipients together with an immunizing dose of *E. coli* bacteria, there were very few PFC on day 4.
The number of PFC per spleen reached a peak on day 6 and remained at the same level on day 8. The number of PFC per $10^6$ spleen cells, on the other hand, had decreased on day 8 (Fig.) 2.

Since the number of PFC per spleen gave more consistent results, especially after transfer, it was decided to express the antibody responses in this way. Day 6 was chosen as the day of assay in the experiments below since the number of PFC was maximal on this day after direct immunization of the normal mice as well as after transfer of spleen cells into irradiated recipients.

![Graph](image)

Fig. 2. The number of PFC per spleen (●) and per $10^6$ spleen cells (○) on various days after the injection of $10 \times 10^6$ normal spleen cells together with $75 \times 10^6$ killed *E. coli* bacteria.

Furthermore, for directly immunized mice the day 6 value is at the end of the PFC peak. If an antibody response in weakly tolerant mice is delayed for 1 or 2 days, as compared to normal mice, but reaches a normal magnitude, it will therefore be detected, if the PFC assay is done on day 6. The reverse is true for the response after transfer. Thus testing for PFC on day 6 reduces the chances of finding a false break in tolerance after a transfer.

**Breaking of Tolerance In Vivo with and without Transfer.**—Tolerant mice were injected with an optimally immunizing dose of *E. coli* bacteria on various days after the last tolerance-maintaining dose of 3 mg of CPS and their antibody responses were tested 6 days later.

It was found that 21 days after the last injection of CPS there was still a
marked depression of the PFC response (Table I). In contrast to this the tolerant state was rapidly broken by transfer of the tolerant cells to irradiated normal recipients (Table II). In general, the transferred spleen cells were taken from mice at the same time as other mice in the same group were tested for

### TABLE I

Persistence of Tolerance without Transfer of Spleen Cells*

| Day of immunization | PFC/spleen | Tolerant mice | Normal mice | P value |
|---------------------|------------|---------------|-------------|---------|
| 2                   | 2.83 ± 0.24 (673)† | 4.65 ± 0.06 (44,100) | <0.001 |
| 3                   | 0.93 ± 0.54 (8) | 4.52 ± 0.10 (33,200) | <0.001 |
| 5                   | 2.01 ± 0.19 (119) | 4.44 ± 0.05 (27,500) | <0.001 |
| 7                   | 3.29 ± 0.20 (1930) | 4.39 ± 0.08 (24,700) | <0.01 |
| 14                  | 1.60 ± 0.54 (40) | 4.19 ± 0.28 (15,500) | <0.01 |
| 21                  | 3.48 ± 0.20 (3020) | 4.29 ± 0.15 (19,300) | <0.05 |

* Tolerant mice were challenged with 75 × 10⁶ *E. coli* bacteria i.v. various days after the last tolerance-maintaining dose of 3 mg of CPS, and the PFC responses were determined 6 days later. Normal mice were immunized at the same time and used as controls. Each group consists of four mice.

† Log₁₀ for the geometric mean ± standard error. Numbers in parentheses = antilogs for the means.

### TABLE II

Breaking of Tolerance after Transfer of Tolerant Spleen Cells

| Day of transfer | PFC/spleen | Tolerant cells | Normal cells | P value |
|-----------------|------------|---------------|-------------|---------|
| 3               | 1.37 ± 0.49 (24) | 3.08 ± 0.11 (1210) | <0.02 |
| 5               | 2.25 ± 0.33 (184) | 3.43 ± 0.05 (2700) | <0.02 |
| 7               | 3.54 ± 0.02 (4470) | 3.32 ± 0.16 (2088) | N.S.* |
| 7               | 3.89 ± 0.16 (7800) | 3.44 ± 0.09 (2763) | N.S. |
| 7               | 3.36 ± 0.04 (2306) | 3.39 ± 0.13 (2449) | N.S. |

Spleen cells were taken from two to three tolerant mice on various days after the last tolerance-maintaining dose of 3 mg of CPS. 10 × 10⁶ of the pooled spleen cells were injected i.v. together with 75 × 10⁶ *E. coli* bacteria into irradiated recipients. Spleen cells from normal mice were used as control. Each group consists of four mice.

* N.S. = not significant.

Breaking of Tolerance by Incubation In Vitro.—Thus when spleen cells were
removed from the tolerant animals, they were able to respond. If this is caused by disappearance of some factors preventing the tolerant cells from responding to the antigen, it should be possible to break tolerance in vitro. This was tested by taking spleen cells from tolerant mice and incubating them in vitro for 20 hr, and thereafter transferring them to irradiated normal recipients (Table III). As controls, spleen cells harvested from tolerant mice in the same group

### TABLE III

#### Breaking of Tolerance by Incubation of Tolerant Spleen Cells In Vitro

| Exp | Cultured cells | Directly transferred cells | P value |
|-----|----------------|---------------------------|---------|
|     | PFC/spleen     | No. of mice               | PFC/spleen | No. of mice |         |
| 1   | 3.27 ± 0.17 (1870) | 4 | 1.45 ± 0.73 (28) | 3 | <0.05 |
| 2   | 2.83 ± 0.19 (679)   | 4 | 0.40 ± 0.40 (3)  | 6 | <0.01 |
| 3   | 2.66 ± 0.55 (461)   | 1 | 0.58 ± 0.58 (4)  | 5 | <0.05 |
| 4   | 2.92 ± 0.10 (828)   | 7 | 1.91 ± 0.32 (82) | 9 | <0.02 |
| 5   | 2.93 ± 0.22 (846)   | 4 | 0.38 ± 0.38 (2)  | 5 | <0.01 |

*10 × 10^6* spleen cells, either cultured in vitro for 20 hr or transferred directly, were injected together with 75 × 10^6 E. coli bacteria. PFC response was assayed 6 days after transfer.

†In experiments 1–3 the cultured cells were taken from tolerant mice 1 day before the directly transferred cells and the transfers for the two groups were done at the same time. In experiments 4 and 5, the spleen cells of the two groups were from the same cell suspension. The transfer and the PFC assay were therefore done 1 day earlier for the group “directly transferred cells” than for the other group.

### TABLE IV

#### Absence of Stimulation of the PFC Response by In Vitro Incubation of Normal Spleen Cells

| Exp | Cultured cells | Directly transferred cells | P value |
|-----|----------------|---------------------------|---------|
|     | PFC/spleen     | No. of mice               | PFC/spleen | No. of mice |         |
| 1   | 3.61 ± 0.08 (4100) | 6 | 3.49 ± 0.43 (3100) | 5 | N.S. |
| 2   | 3.17 ± 0.14 (1480) | 3 | 3.11 ± 0.17 (1270) | 5 | N.S. |
| 3   | 3.37 ± 0.08 (2350) | 5 | 3.52 ± 0.12 (3310) | 5 | N.S. |

*10 × 10^6* normal spleen cells, either cultured 1 day in vitro or transferred directly, were injected i.v. together with 75 × 10^6 E. coli bacteria into irradiated recipients and the PFC response was determined 6 days later.

were transferred directly after one washing without incubation in vitro. In experiments 1–3 the control cells were removed from tolerant mice 1 day after the cultured cells and transferred at the same time as the latter. Thus, the total time lapse from the last tolerance-maintaining dose and the assay for antibody production was the same for the two groups. In experiments 4 and 5, the directly transferred cells and the incubated cells were from the same cell suspension. It was then necessary to do the transfer and the antibody assay 1 day earlier in the control group. It was found that a substantial reactivation of the tolerant cells could be obtained by the incubation in vitro.
To exclude the possibility that a small fraction of nontolerant cells for some reason is activated, and gives much higher numbers of PFC per immunocompetent cell after incubation in vitro, normal cells were incubated for 20 hr and their PFC response against CPS after transfer was compared to that of normal cells transferred without in vitro incubation. The experiments show (Table IV) that nonincubated cells respond as well as incubated cells, thus making the alternative given above unlikely.

**DISCUSSION**

It has been suggested that persistence of tolerance is dependent on the presence of antigen (1). If administration of the paralyzing antigen is stopped, the animals will ultimately regain immunological reactivity. The spontaneous recovery from tolerance was not fully investigated in this study, but Britton earlier found that mice receiving a single dose of 10 mg of CPS did not respond normally until 36 days after the injection of the paralyzing antigen (22). However, if tolerant spleen cells are removed from the antigen-containing environment, a significant immune response can occur earlier. Thus, a normal immune response was achieved when the tolerant spleen cells were transferred into irradiated recipients 5 or 7 days after the last tolerance-maintaining dose of CPS. It was also possible to break tolerance partially by incubating cells, taken from tolerant mice 3 or 4 days after the last tolerance-maintaining dose, for 20 hr in vitro before transfer. Tolerant cells transferred without incubation did not respond at this time. The breaking of tolerance in vitro was not due to an augmented response by a few nontolerant cells, since normal cells treated in the same way did not show an increased response.

Two alternatives are possible to explain the results: (a) tolerance is broken by recruitment of new immunocompetent cells from stem cells; (b) tolerant immunocompetent cells are reactivated.

Recruitment of new immunocompetent cells is an unlikely possibility, since the antigenic stimulation occurred at the time of cell transfer and the assay for PFC was done at the beginning of the peak of the antibody response, thus leaving no time for stem cells to differentiate in the recipients. Furthermore, it seems highly unlikely that such regeneration of immunocompetent cells could occur during the incubation in vitro. Thus, removal of tolerant cells from the tolerant animals brings about a reactivation of the cells. The breaking of tolerance in vitro speaks for a diffusing away of a tolerizing factor from the cells as the reactivation mechanism. This factor seems most likely to be the antigen, but antibodies against the antigen or against antigen-receptor complexes are other possibilities which are suggested by recent results (38) and cannot be ruled out.

In addition, the transfer of the cells into irradiated recipients may also play

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a minor role. Sublethal irradiation can abolish tolerance in partially tolerant animals (23–26). Furthermore, sera from irradiated mice contain a factor which will stimulate an immune response in vitro (Cooperband, personal communication). In fact, Dresser (27) has suggested that the general cell proliferation occurring after a transfer leads to a dilution of the tolerizing antigenic determinants within the inactivated cells, thereby making them able to respond to the antigen again.

There are other recent results which are compatible with the reactivation of tolerant cells. Howard and colleagues (personal communication) have been able to partially break tolerance to pneumococcal polysaccharide by transfer of the tolerant cells into irradiated recipients. In one-third of their experiments, McCullagh and Gowans (5) were able to reactivate thoracic duct lymphocytes tolerant against sheep red cells by incubating them overnight in vitro. Byers and Sercarz (7) induced tolerance in vitro by large doses of bovine serum albumin. By removing the tolerizing antigen they were able to obtain a normal, but retarded, response. It is not clear yet what relevance such a short-term tolerance has for the events occurring in an intact animal. Finally, Cohen et al. (8) could induce autosensitization by incubating lymphoid cells in vitro for 5 days with syngeneic fibroblasts. The finding that a graft-versus-host reaction can produce a short-lived immune response in rats tolerant to sheep red cells is another indication of the presence of immunocompetent cells in tolerant animals (6). In this latter case, it could be that immunocompetent bursa-equivalent lymphocytes (B cells) exist in the tolerant rats and that the graft-versus-host reaction serves as a helper mechanism, substituting for the tolerant thymus-derived lymphocytes (T cells) in analogy with results of Katz et al. (28).

Thus, tolerance both to CPS and to pneumococcal polysaccharide can be broken by a transfer of the tolerant cells into irradiated recipients. One explanation for this may be that an increased number of antigen-binding cells, as compared to normal unimmunized mice, exist in animals tolerant to CPS as well as to pneumococcal polysaccharide. Possibly part of these represent reversibly inactivated immunocompetent cells (10). If this is so, it follows that tolerance can be induced in cells which have already been triggered by the antigen. Whether nontriggered tolerant cells can also be reactivated in the same way cannot be decided at the moment.

Another explanation for the ease with which tolerance is broken is that both CPS and pneumococcal polysaccharide are thymus-independent antigens (29–31). Tolerance is more easily induced and more sustained in the T cell than in the B cell population (32–34). In accordance with this, tolerance to the thymus-dependent antigen human gamma globulin (35) has been shown to persist for a prolonged period, also after transfer (36). Furthermore, spontaneous recovery from tolerance against thymus-dependent antigens is markedly delayed in thymectomized mice (2, 3). These findings suggest that T cells, to a larger extent than B cells, are irreversibly inactivated or killed by tolerance
induction. It may be that T cells, because of their recirculation (37), are more easily eliminated than B cells, which are regarded to be stationary in the lymphoid tissues to a larger extent.

SUMMARY

The breaking of tolerance against the lipopolysaccharide from *E. coli* 055:B5 was studied. It was found that immune responsiveness recovered very slowly in vivo, tolerance still existing 3 wk after the last tolerizing injection. However, if spleen cells from tolerant mice were transferred into irradiated syngeneic recipients, the tolerant state was readily broken. Spleen cells transferred 3 days after the last tolerance-maintaining dose did not respond, whereas cells transferred on day 5 or 7 responded equally well as normal spleen cells. It was also possible to break tolerance by incubating tolerant spleen cells, which did not respond after transfer, for 20 hr in vitro before transfer into irradiated recipients. The results suggest that there exist reversibly inactivated cells in tolerant animals and that these cells can be reactivated upon removal of the cells to a neutral environment.

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