REGULATION OF ANTIBODY SYNTHESIS AGAINST ESCHERICHIA COLI ENDOTOXIN

IV. Induction of Paralysis In Vitro by Treating Normal Lymphoid Cells with Antigen*

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A variety of experimental data (see Discussion) suggests that the direct interaction between antigen and the specifically reactive lymphoid cells leads to paralysis, whereas the induction of immunity requires some antigen-processing steps (1). However, experiments aimed at proving conclusively that direct access of antigen to susceptible cells results in paralysis by exposing suspensions of normal lymphoid cells from different rodents to high doses of various heterologous serum proteins in vitro, and thereafter testing the reactivity of such treated cells in a secondary irradiated host, have been entirely unsuccessful (e.g., R. T. Smith, personal communication and reference 2).

Recently, however, Diener and Armstrong (3) reported that normal mouse spleen cells in tissue culture could be made specifically unresponsive after exposure to high doses of the polymeric form of flagellin from *Salmonella adelaide*.

The failure to induce paralysis in vitro with heterologous serum proteins is in contrast to the successful induction of paralysis when these antigens are administered in certain dose ranges in vivo. Polymeric flagellin, however, in contrast to the monomeric form, cannot readily be used as a paralytogen in vivo, whereas it is quite efficient in inducing paralysis in vitro. The discrepancies between these two categories of antigens in these respects cannot be adequately explained at present.

Endotoxins of Gram-negative bacteria are powerful immunogens in many rodents (4, 5) and after detoxification they can be injected in doses which will induce specific paralysis in adult animals (6, 7). Endotoxins that have been detoxified with alkali have a marked tendency to attach to cell membranes (8) and, therefore, it was important to know whether such material would be competent to paralyze lymphoid cells exposed to the antigen in vitro. This report

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deals with the immunological consequences of treating normal mouse lymphoid cells in vitro with various doses, and under different conditions, of detoxified *Escherichia coli* 055:B5 endotoxin.

Materials and Methods

Animals.—6–8 wk old inbred male CBA mice were used throughout the experiments.

Antigens.—Lipopolysaccharide of *E. coli* 055:B5 was obtained and detoxified with mild alkali (8) as described previously (6). ^{14}C-labeled lipopolysaccharides were obtained by phenol extraction from whole bacteria that had been grown in a synthetic medium where ^{14}C-glucose (D-glucose-^{14}C [CFB2] The Radiochemical Centre, Amersham, England) was the carbon source. Crystallized human serum albumin (HSA) was purchased from Kabi, Stockholm, Sweden. Sheep red blood cells (SRBC) washed three times in a balanced salt solution (BSS) were used in the in vitro techniques and for immunizations (1×10^8 SRBC/mouse). Unless otherwise stated, heat-inactivated bacterial vaccine of *E. coli* 055:B5 (4) was used for in vivo immunizations (0.1 ml of a suspension containing 750×10^5 organisms/mouse). All immunizations were made intravenously.

The Agar Plaque Technique.—Detection of individual plaque-forming cells (PFC) producing antibody against SRBC or SRBC coated with *E. coli* endotoxin was performed as described previously (4) except that the agar was exchanged for agarose (from l'Institut Biologique Française, Gennevilliers, France) according to Wortis et al. (9). Only direct (19S) PFC were investigated.

Serological Procedures.—Hemolysins and agglutinins were determined as described previously (4).

Irradiation.—X-rays were generated in a Siemens X-ray machine at 200 kv and 15 ma and were filtered by 1 mm Cu for whole body irradiation and by 1 mm Al for irradiation of cell suspensions. All secondary hosts were irradiated with 750–900 R at a rate of 109 R/min. Cells were irradiated with 3000 R at a rate of 196 R/min.

Preparation of Cell Suspensions.—Spleen, inguinal, and axillary lymph node cell suspensions were prepared by pressing the organs through a 60 mesh stainless steel screen and suspending the cells in Eagle's medium in Earle's solution. Spleens were perfused with the same medium before being brought into suspension. The cell suspensions were kept in an ice bath and washed three times in cold Eagle's solution.

Experimental Procedure.—The in vitro treatment of the lymphoid cells with antigen was performed at various temperatures and for various time periods (see Results). Different concentrations of the detoxified endotoxin diluted in BSS were added to the cells as described below, but the standard dose of antigen which induced paralysis under optimal in vitro conditions was 100 μg/10^6 lymphoid cells. Equal volumes of antigen solution and cell suspensions were used for the in vitro treatment, and the total volume was between 8 and 12 ml. The cell concentration during the in vitro incubations was generally 100×10^6 cells/ml. The incubations at 37°C were carried out with constant agitation by a magnetic stirrer, those at 4°C with repeated manual agitations. The pH was adjusted to neutrality before and after incubation. After incubation, the cells were spun down, washed 5–7 times in ice cold Eagle's solution, and made up to the initial volume in the same medium. The number of viable cells before and after incubation was determined by the trypan blue exclusion technique. Quantitation of the uptake of antigen on the lymphoid cells was made by determining the amount of ^{14}C-endotoxin left on the cells after the incubation procedure and washing. Various concentrations of ^{14}C-endotoxin dissolved in BSS were added to a fixed amount of cells as above. After incubation, the cells were spun down and washed five times in 20 ml volumes of BSS. After the washings, the cellular sediments were dissolved in 1 ml of concentrated formic acid. 0.3 ml of this solution was added to plastic planchettes which were dried and subsequently

counted in a gas flow Geiger detector (Nuclear-Chicago, Des Plaines, Ill.). 0.3 ml from each washing solution was also dried and counted accordingly.

Trypsinization of lymphoid cells was performed by exposing freshly harvested cells for 90 min at 37°C to a 0.25% solution of trypsin (State Bacteriological Laboratory, Stockholm) diluted in BSS. Equal volumes of trypsin solution and cells were mixed and constantly stirred during incubation. After this procedure the cells were washed three times in cold Eagle's solution and made up to the initial concentration.

After antigen treatment, the cells were washed five times and transferred into the irradiated secondary hosts which were syngeneic with the cell donor. 30–50 $\times$ 10^6 cells in 0.2 ml were injected intravenously. The recipients were immunized 12 hr after cell transfer and their immune status was tested at various times thereafter.

RESULTS

It is well established that a primary as well as a secondary immune response can be induced in lethally or sublethally irradiated recipients repopulated with

TABLE I

*The Effect of Exposure of Normal Mouse Lymphoid Cells to Different Doses of Detoxified E. coli 055:B5 Endotoxin and Human Serum Albumin (HSA) on the Viable Cell Count**

Antigen treatment	No. of viable cells added	No. of viable cells recovered	Reduction in viability
			%
100 μg endotoxin/10^6 cells	500 $\times$ 10^6	398 $\times$ 10^6	20.4
10 μg endotoxin/10^6 cells	500 $\times$ 10^6	412 $\times$ 10^6	17.6
100 μg HSA/10^6 cells	500 $\times$ 10^6	356 $\times$ 10^6	28.2
10 μg HSA/10^6 cells	500 $\times$ 10^6	380 $\times$ 10^6	24.0
None	1000 $\times$ 10^6	848 $\times$ 10^6	15.2

* Exposure, 120 min at 37°C. Equal volumes of cells and antigen solution were used. Cells were washed five times after antigen treatment. Cell count determined by supravital staining with trypan blue.

‡ Incubated without antigen for 120 min at 37°C. Washed five times.

normal or presensitized isogeneic lymphoid cells, respectively. If the test for immunity is made shortly after irradiation and cell transfer, the response can be attributed to the transferred cells, since unrepopulated recipients will not respond to antigenic challenge (10).

Specificity of Paralysis Induced by Antigen Treatment of Lymphoid Cells in Vitro.—Although detoxified lipopolysaccharides of *E. coli* 055:B5 still retain a certain degree of toxicity in vivo (6), they did not appear to be especially harmful to lymphoid cells in vitro as judged by the trypan blue exclusion technique (Table I).

Furthermore, lymphoid cells that have been exposed to endotoxin in a dose of 100 μg/10^6 lymphoid cells in vitro for 120 min, washed, and thereafter transferred to irradiated hosts which were injected subsequently with SRBC, re-

sponded normally to this antigen at the cellular and at the serum level as well (Fig. 1). The kinetics of the primary response to SRBC and to *E. coli* endotoxin in the present test system differed from that obtained after in vivo immunization, since the peak response in the irradiated repopulated host occurs at day 7–8, as compared with day 4–5 when the antigen is injected into normal mice.

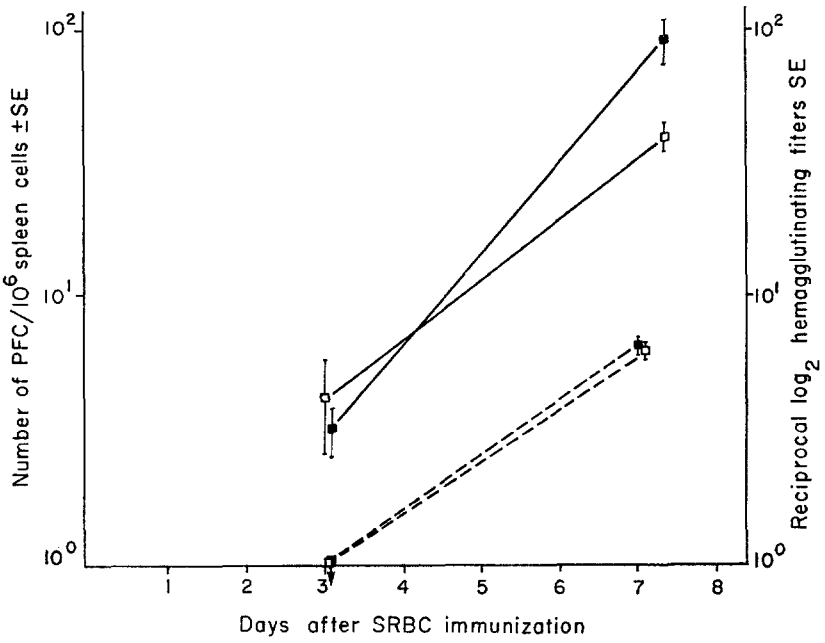

FIG. 1. Number of PFC/10^6 spleen cells ± SE and hemagglutinin titers ± SE in lethally irradiated (900 R) mice repopulated with 50 × 10^6 syngeneic lymphoid cells and, 12 hr after irradiation and repopulation, injected with 1 × 10^8 SRBC intravenously. □——□ indicates PFC and □----□ hemagglutinins in mice repopulated with spleen and lymphoid cells incubated in vitro without antigen for 120 min at 37°C. ■——■ indicates PFC and ■-----■ hemagglutinins in mice repopulated with cells incubated in vitro with 100 μg endotoxin/10^6 cells for 120 min at 37°C and thereafter washed.

In contrast, endotoxin-treated lymphoid cells from the same batch as those in Fig. 1 but subsequently transferred to irradiated hosts, which were injected with an *E. coli* bacterial vaccine 12 hr later, did not respond with cellular or humoral antibody synthesis (Fig. 2). Since cells incubated in the absence of antigen for 120 min at 37°C respond to both *E. coli* and SRBC after transfer into secondary irradiated hosts to the same extent as nonincubated lymphoid cells (Table II), it seems likely that normal mouse spleen and lymph node cells

can be made specifically unresponsive by in vitro treatment with *E. coli* endotoxin.

The Dose of Antigen Needed for Induction of Paralysis In Vitro.—Attempts were made to determine the concentration of antigen necessary for induction of paralysis in vitro by exposing lymphoid cells to various concentrations of antigen for 120 min at 37°C. After five washings, the treated cells were trans-

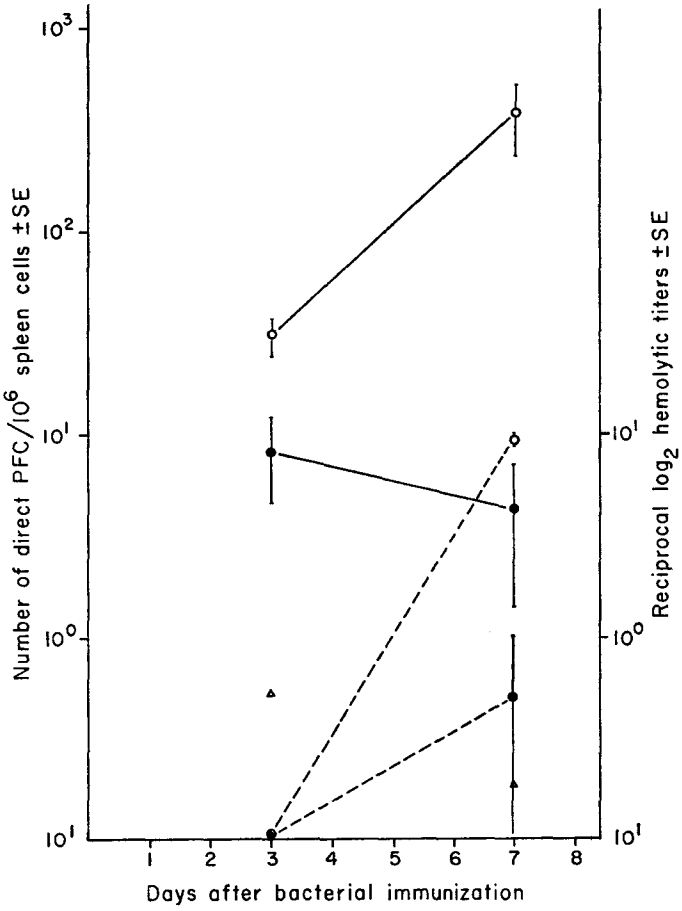

FIG. 2. Number of PFC/10^6 spleen cells ± SE and hemolytic titers ± SE in lethally irradiated (900 R) mice repopulated with 50 × 10^6 syngeneic lymphoid cells and, 12 hr after irradiation and repopulation, injected with bacterial vaccine (75 × 10^6 organisms) intravenously. O——O indicates PFC and O----O hemolytic titers in mice repopulated with spleen and lymphoid cells incubated in vitro without antigen for 120 min at 37°C. ●——● indicates PFC and ●----● hemolytic titers in mice repopulated with cells incubated in vitro with 100 μg endotoxin/10^6 cells for 120 min at 37°C and thereafter washed. △ indicates number of PFC in nonrepopulated animals.

ferred into irradiated hosts, which were immunized 1 day later with a bacterial vaccine or, alternatively, left untreated. The number of 19S PFC was determined 7 days after immunization (Table III). As can be seen, cells treated with 100 μg endotoxin/10^6 cells did not respond with antibody synthesis to a

TABLE II

*Number of PFC/10^6 Spleen Cells ± SE in Irradiated CBA Mice (750 R), Repopulated with 50 × 10^6 Syngeneic Lymphoid Cells and 12 Hr Later Injected with SRBC and E. coli Bacteria, Respectively**

Antigen treatment	Day 4‡		Day 6‡		Day 8‡	
	Arithmetic mean number of PFC/10^6 spleen cells ±SE		Arithmetic mean number of PFC/10^6 spleen cells ±SE		Arithmetic mean number of PFC/10^6 spleen cells ±SE	
	Anti-SRBC§ PFC	Anti-*coli*‖ PFC	Anti-SRBC§ PFC	Anti-*coli*‖ PFC	Anti-SRBC§ PFC	Anti-*coli*‖ PFC
SRBC	24.0 ± 8.4	Not done	88.8 ± 24.0	Not done	128.6 ± 34.8	Not done
Bacteria	12.6 ± 6.0	18.6 ± 10.0	21.7 ± 12.4	104.0 ± 24.6	19.6 ± 11.4	140.0 ± 48.6
None¶	5.6	8.8	4.2	12.2	8.8	16.1

* 1 × 10^8 SRBC in 0.2 ml intravenously. 75 × 10^6 heat-killed *E. coli* organisms intravenously. Four to five mice per group.

‡ Days after immunization.

§ Tested against SRBC.

‖ Tested against endotoxin-sensitized SRBC.

¶ One mouse per test day.

TABLE III

*The Effect of Dose on the Ability of Endotoxin to Paralyze Lymphoid Cells In Vitro**

Dose of endotoxin added in vitro	Arithmetic mean number of PFC/10^6 spleen cells ± SE	Mean $\log_2$ agglutinin titer ± SE
100 μg/10^6 cells	1.5 ± 1.2	0
10 μg/10^6 cells	86.4 ± 32.4	3.4 ± 1.6
1 μg/10^6 cells	450.0 ± 212.4	3.6 ± 0.8
0.001 μg/10^6 cells	134.5 ± 58.4	4.4 ± 1.8
None‡	121.1 ± 72.3	5.6 ± 1 8

* Cells incubated 120 min at 37°C. Washed five times. 40 × 10^6 cells transferred to secondary irradiated (750 R) hosts immunized (75 × 10^6 heat-killed bacteria) 12 hr after cell transfer. Test at day 7 after immunization. Four to five mice per group.

‡ Cells incubated without antigen for 120 min at 37°C.

subsequent injection with bacterial vaccine. Cells exposed to 10 μg/10^6 cells responded subnormally, whereas those treated with 1 μg/10^6 exhibited a normal immune response.

Estimations on the Amount of Antigen Transferred with the In Vitro–Treated Cells.—It is essential to determine the amount of antigen left on the lymphoid cells after the in vitro treatment and the washing procedure, in order to dif-

ferentiate between induction of paralysis in vitro and the possibility that sufficient amount of antigen was transferred with the cells into the recipients to induce paralysis in vivo.

Previously (11) this was attempted by determining the retained radioactivity

TABLE IV

*The Amount of Antigen Retained on Normal Lymphoid Cells after Treatment In Vitro for 120 Min at 37°C with Various Doses of ^{14}C-Endotoxin**

Amount of ^{14}C-endotoxin added	Activity‡ in supernatant§	Activity‡ in first washing	Activity‡ in second washing	Activity‡ in third washing	Activity‡ in fourth washing	Activity‡ retained on the cells	Activity retained
							%
100 μg/10^6 cells	1,848,811	45,655	4,930	2,482	2,171	5,588	0.29
10 μg/10^6 cells	228,897	4,296	1,603	673	706	1,236	0.55
1 μg/10^6 cells	32,280	739	409	455	270	220	0.64
0	547	165	1,008	481	262	445	—

* 500 × 10^6 washed spleen cells incubated with various concentrations of ^{14}C-endotoxin diluted in BSS. Total incubation volume 10 ml. After incubation the cells were washed four times in 20 ml BSS.

‡ Counts per minute. Mean of two planchettes. Background substracted.

§ Supernatant obtained after spinning down the incubated cells.

TABLE V

*The Ability of 50 × 10^6 Endotoxin-Treated, Irradiated CBA Lymphoid Cells to Immunize A/Sn Recipient Mice against E. coli Endotoxin**

Treatment of transferred cells	Day 3‡	Day 5‡	Day 5‡
	Arithmetic mean number of PFC/10^6 spleen cells ± SE	Arithmetic mean number of PFC/10^6 spleen cells ± SE	Mean $\log_2$ agglutinin titer ± SE
100 μg endotoxin/10^6 cells	36.8 ± 12.0	89.6 ± 21.4	3.6 ± 1.2
10 μg endotoxin/10^6 cells	44.0 ± 8.8	11.0 ± 4.8	1.2 ± 0.2
1 μg endotoxin/10^6 cells	2.1 ± 0.8	4.6 ± 2.4	0
None	0.8 ± 0.2	1.2 ± 0.8	0

* Treated with various doses of endotoxin for 120 min. Washed five times. Five mice per group per test day.

‡ Days after immunization.

on cells that had been exposed to an ^{51}Cr-labeled endotoxin. However, this procedure had serious drawbacks since the labeling of the endotoxin with the very acid ^{51}Cr-Cl_3 solution changed both the ability of the endotoxin to sensitized red cells as well as decrease its immunogenicity in vivo (S. Britton, unpublished data). These difficulties were overcome by using internally ^{14}C-labeled endotoxin. This label is stable and does not affect the sensitizing or

immunogenic properties of the endotoxin. Various amounts of the ^{14}C-labeled endotoxin were added to a fixed amount of mouse lymphoid cells and the mixtures were incubated for 120 min at 37°C. Thereafter, the cells were washed five times in calibrated volumes of BSS and the radioactivity retained on the cells was determined as well as the radioactivity in the different washing solutions. As can be seen from Table IV, less than 1 % of the added labeled material was retained on the cells after such incubation and washing procedure, which means that when cells have been treated with a paralyzing concentration of endotoxin approximately 5–10 μg endotoxin is transferred with the cells to the secondary hosts, a dose that is highly immunogenic in vivo (6).

Other approaches were also used to evaluate the amount of antigen left on

TABLE VI

*The Effect of Addition of 50 × 10^6 Endotoxin-Treated Irradiated Cells on the Ability of 50 × 10^6 Normal Lymphoid cells to Restore the Immune Capacity of Secondary Irradiated Hosts (750 R) against E. coli**

Cells transferred	Arithmetic mean number of PFC/10^6 spleen cells ± SE	Mean $\log_2$ hemagglutinating titer ± SE
Endotoxin-treated irradiated and normal	180.4 ± 34.6	4.2 ± 1.6
Normal	138.6 ± 38.6	5.2 ± 2.8
Endotoxin-treated	2.4 ± 1.6	0
Endotoxin-treated irradiated	3.6 ± 0.8‡	0

* Added cells treated with 100 g endotoxin/10^6 cells for 120 min and washed five times. Lymphoid cells incubated for 120 min without antigen. 75 × 10^6 heat-killed bacteria injected intravenously 12 hr after transfer. Tested 7 days after immunization. Eight mice per group.

‡ Mean of four mice.

the cells after antigen treatment. Thus, cells from CBA mice were treated with various doses of endotoxin for 120 min at 37°C, irradiated with 3000 R, and thereafter transferred into H-2 incompatible (A/Sn) mice (50 × 10^6 cells/mouse). As seen in Table V, cells treated with 100 μg and 10 μg endotoxin/10^6 cells evoked an immune response against the endotoxin in the secondary hosts. Thus, the amount of antigen adsorbed to the cells was sufficient to initiate a primary response in a nonirradiated incompatible host, but insufficient to induce paralysis.

The conditions favoring the induction of paralysis may, however, be different in unirradiated and irradiated repopulated animals. In order to test this and mimic the above conditions leading to paralysis as closely as possible, cells which had not been incubated with antigen in vitro were transferred to irradiated hosts, in a mixture with irradiated lymphoid cells (50 × 10^6 cells/mouse) that had been exposed in vitro to the dose of antigen that results in

paralysis. 12 hr after cell transfer, the animals were challenged with bacterial vaccine. The rationale for this approach was to investigate whether cells exposed to a paralyzing dose of antigen in vitro would transfer such a large amount of antigen or antigen presented in such a way that paralysis could be induced in a simultaneously transferred nonantigen-treated cell population. As can be seen in Table VI, the simultaneous injection of antigen-treated and nonantigen-treated cells did not lead to paralysis in the nontreated transferred cells. Considered together, the findings suggest that the amount of antigen transferred with the cells exposed to 100 μg endotoxin/10^6 cells in vitro was insufficient to induce paralysis by itself in nonirradiated hosts or in untreated lymphoid cells injected simultaneously into irradiated hosts. It seems likely, therefore, that the in vitro exposure of the lymphoid cells to antigen was the relevant variable for the processes eventually leading to paralysis, the dose of

TABLE VII

*The Effect of Various Incubation Conditions on the Ability of Endotoxin (100 μg/10^6 cells) to Paralyze Normal Lymphoid Cells In Vitro**

Time of incubation	Temperature at incubation	Arithmetic mean number of PFC ± SE‡	Meanlog$_2$ agglutinin titer ± SE‡
min	°C		
120	37	3.8 ± 1.8	0
120	4	27.9 ± 8.2	1.6 ± 0.6
12	37	98.8 ± 12.0	4.4 ± 1.2
12	4	112.0 ± 24.8	5.0 ± 2.4

* 50 × 10^6 cells transferred to secondary irradiated (800 R) hosts.

‡ Tested 7 days after injection of 75 × 10^6 heat-killed bacteria. Eight mice per group.

antigen transferred being clearly immunogenic if given directly to intact animals.

Variables Affecting Induction of Paralysis In Vitro.—Another approach aiming at a distinction between the induction of paralysis in vitro or in vivo was to vary the incubation conditions. Lymphoid cells were incubated with the same concentration of antigen (100 μg/10^6 cells) for varying time periods (12 or 120 min) and at different temperatures (4° or 37°C) and their immunological competence tested in irradiated recipients as before. As seen in Table VII, incubation for 120 min at 37°C resulted in a marked suppression of the number of PFC in the secondary hosts, whereas treatment for 120 min at 4°C only caused a limited degree of paralysis. Cells treated for 12 min at 4° or 37°C responded equally well as untreated cells.

Effect of Trypsin on Induction of Paralysis In Vitro.—Cells treated with 100 μg endotoxin/10^6 in vitro became unreactive to the endotoxin but responded normally to SRBC (Figs. 1 and 2). It is likely, therefore, that only the fraction

of cells containing the receptors for the appropriate fixation of endotoxin are eliminated or made unresponsive. By eliminating the receptor material on the cell surface it would seem possible to prevent the specific interaction between antigen and cells and, therefore, to prohibit the induction of paralysis.

It seems likely that the surface receptors on lymphoid cells are of immunoglobulin nature. Therefore, cells were treated with the proteolytic enzyme trypsin for 90 min immediately before mixing them with a paralyzing dose of the endotoxin. Table VIII lists the data obtained with trypsinized and nontrypsinized cells treated with antigen in vitro and thereafter transferred into secondary irradiated hosts. As can be seen, the trypsinized cells responded as untreated cells in the secondary hosts, although they had been subjected to the same in vitro treatment with antigen that resulted in paralysis of the nontrypsinized cells.

TABLE VIII

*The Effect of Pretreatment with Trypsin on the Ability of Endotoxin (100 g/10^6 Cells) to Paralyze Lymphoid Cells In Vitro**

Trypsinization	Endotoxin treatment‡	Arithmetic mean number of PFC/10^6 spleen cells ± SE	Mean $\log_2$ hemolytic titer ± SE
Yes	Yes	137.2 ± 48.2	8.6 ± 2.4
Yes	No§	80.0 ± 24.6	8.6 ± 0.8
No‖	Yes	4.0 ± 2.6	1.8 ± 0.8
No‖	No§	72.9 ± 18.6	6.0 ± 2.8

* Cells (40×10^6) transferred to secondary irradiated (750 R) hosts 12 hr later injected with 75×10^6 heat-killed bacteria. Tested 7 days after immunization. Eight mice per group.

‡ 100 μg endotoxin/10^6 cells for 120 min at 37°C. Cells washed five times.

§ Incubated without endotoxin for 120 min at 37°C. Washed five times.

‖ Incubated without trypsin for 90 min at 37°C. Washed three times.

DISCUSSION

These experiments have demonstrated that normal mouse lymphoid cells can be specifically paralyzed by a brief exposure to high doses of *E. coli* endotoxin antigen in vitro, as judged by their failure to specifically restore the immune capacity of irradiated secondary hosts.

According to one hypothesis, paralysis is induced in the immunocompetent cells when they are directly confronted with antigen, whereas contact between the cells and antigen fixed onto other cell types or processed in some way would favor immunity (1). So far, the direct interaction hypothesis has been based on constancy with regard to the doses leading to paralysis against a variety of soluble antigens, which is in marked contrast to the irregularity of the doses giving rise to an immune response to the same antigens. This has been taken as evidence for the possibility that induction of paralysis is a simple process

(direct access) as compared to the more complex induction of immunity. Furthermore, when a heterologous protein is centrifuged in order to eliminate aggregated material (12) or *screened in vivo* by drawing serum from rabbits previously injected with a heterologous serum protein so as to obtain only the fraction of the injected material, which is not rapidly eliminated from the circulation by phagocytosis (13), the material is much more competent to induce paralysis than the original preparations. However, attempts to demonstrate that direct exposure of lymphoid cells to high doses of protein antigens in vitro would result in paralysis have been unsuccessful (reference 2, and R. T. Smith, personal communication). Recently, however, Diener and Armstrong (3) found that mouse lymphoid cell suspensions exposed in vitro to high doses of polymeric flagellin from *S. adelaide* were made unresponsive, as shown by their failure to induce a primary immune response in the culture after removal of excess antigen and by the incompetence of the pretreated cells to specifically restore the immune capacity of irradiated secondary hosts. A different system was used by Scott and Waksman (14). These workers showed that cells taken from intact lymphoid organs, previously injected with high doses of bovine γ-globulin in vitro, were specifically unresponsive when transferred into thymectomized, irradiated secondary hosts, as judged by their inability to produce 7S agglutinins when stimulated by the antigen incorporated in Freund's adjuvant. These authors were unable to obtain a similar effect when the lymphoid cell suspensions were treated with high doses of antigen in vitro. Therefore, they concluded that close cell-to-cell contact, as well as some antigen-processing steps occurring in the intact lymphoid organs, was necessary for the induction of paralysis and antibody formation as well.

The present experiments indicate that lymphoid cell suspensions can be paralyzed by exposure to high doses of detoxified *E. coli* endotoxin in vitro. Paralysis was dose dependent: 100 $\mu g/10^6$ cells leading to paralysis, whereas 10 $\mu g/10^6$ was less effective in this respect. Quantitative estimations of the amount of antigen remaining on the cells after the in vitro treatment indicated that only a minor fraction ($< 1\%$) of the added antigen was retained; various control experiments indicated that unresponsiveness was initiated in vitro, and was not caused by transfer of antigen into the secondary hosts, where actual induction occurred. Thus, cells which were treated with a paralyzing dose of antigen in vitro and thereafter irradiated were found to be unable to induce paralysis in a non-antigen-treated cell population, which was injected simultaneously into the secondary hosts. Furthermore, paralysis was not induced when the cells were exposed to antigen for a short period (12 min) or at low temperature (4°C). The latter findings are in contrast to the results obtained with flagellin (G. J. V. Nossal, personal communication) where the cells could be equally well paralyzed upon exposure with the antigen in the cold.

The reason for the discrepancy between the ability of heterologous proteins

and the endotoxin antigens to paralyze cells in vitro remains unknown. One possible reason for the effectiveness of endotoxins in this respect could be the high affinity of these substances for cell membranes, as illustrated by the ability of endotoxin in high concentrations to sensitize mouse lymphoma cells for cytolysis by specific antibodies to the endotoxins (S. Britton, unpublished data). However, Scott and Waksman (14) showed that a substantial fraction of the BGG added to lymphoid cell suspensions in vitro remained on the cells after the washing procedure and yet paralysis was not induced. The author (unpublished data) has tried to paralyze mouse lymphoid cells by exposing them to human γ-globulin containing anti-mouse antibodies which facilitated the uptake of the heterologous γ-globulin. However, the cells did not become unresponsive in the secondary host by this procedure.

The demonstration that cells from lymph nodes injected with bovine γ-globulin were specifically unable to produce 7S agglutinins, whereas exposure of dispersed lymph node cells to the same amount of antigen did not lead to paralysis (14), suggests that suppression of 7S synthesis is more complex and that paralysis in this case requires interference with certain antigen-possessing steps as well as with the immunocompetent cells. Endotoxins and flagellin stimulate a vigorous primary 19S synthesis, which may represent a more primitive immune response, and therefore may be more easily amenable to suppression by direct antigen interaction with the competent lymphocytes.

The specificity of unresponsiveness obtained after in vitro treatment of lymphoid cells, both with regard to *E. coli* endotoxin, flagellin (3), and bovine γ-globulin (14), suggests that only the specifically reactive cells were inactivated by the direct exposure with these antigens. It seems probable that the antigen-specific receptors are immunoglobulinic, and it is known that surface-bound γ-globulin can be digested away by trypsin (15, 16). The susceptibility of sensitized lymphoid cells to inhibition of migration after contact with specific antigen in the macrophage outgrowth test for delayed hypersensitivity is abolished if the sensitized cells are pretreated with trypsin (17). It is possible that the specific interaction between antigen and sensitive lymphocyte in vitro was abrogated by the enzymatic removal of γ-globulins on the lymphoid cells. In the present system an analogous approach was attempted by pretreating the lymphoid cells with trypsin and thereafter study their susceptibility to the induction of paralysis. Trypsinized cells did not become paralyzed by antigen exposure in vitro. However, it can be argued that trypsinized cells do not take up as much antigen as nontrypsinized cells and, therefore, less antigen would be transferred into the irradiated host by the trypsinized cells. This possibility has not yet been ruled out, nor has it been excluded that the trypsinized cells will "home" at sites different from the nontrypsinized cells in the secondary host (18) and that this primary localization (e.g., liver vs. spleen) is of importance in the handling of the antigen retained on the cells.

These experiments suggest, however, that direct interaction between antigen and normal lymphoid cells may result in specific induction of paralysis in the 19S responding cells and that paralysis is a process requiring high doses of antigen as well as specific incubation conditions. The latter findings suggest that the binding between the paralytogen and the susceptible cells results from some metabolic processes. Whether paralysis is caused by the killing of the competent cells or by active induction in the cells without their actual elimination has not been clarified by these experiments. However, the addition of complement to the in vitro incubation mixture of cells and antigen does not facilitate the induction of paralysis (S. Britton, unpublished data).

SUMMARY

Normal mouse lymphoid cells have been shown to become specifically paralyzed after in vitro exposure to high doses of detoxified endotoxin of *Escherichia coli* 055:B5. The immune status of the treated cells was tested after transfer to secondary irradiated hosts. Paralysis was shown to be initiated by events taking place in vitro, since the amount of antigen retained on the cells after the in vitro exposure was insufficient to induce paralysis in vivo. The induction of paralysis was dependent on the concentration of antigen added to the cells in vitro. Certain variables, such as time of exposure and temperature at exposure, influenced the ease by which the cells could be paralyzed. Cells pretreated with trypsin were not susceptible to induction of paralysis by the above procedure.

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