SPLEEN CELLS FROM ANIMALS TOLERANT TO A THYMUS-DEPENDENT ANTIGEN CAN BE ACTIVATED BY LIPOPOLYSACCHARIDE TO SYNTHESIZE ANTIBODIES AGAINST THE TOLEROGEN*

By GÖRAN MÖLLER, EVA GRONOWICZ, ULLA PERSSON, ANTONIO COUTINHO, ERNA MÖLLER, LENNART HAMMARSTRÖM, and EDVARD SMITH

(From the Division of Immunobiology, Karolinska Institute, Wallenberglaboratory, Lilla Freskati, 104 05 Stockholm 50, Sweden)

It is generally accepted that tolerance to thymus-dependent antigens affects both T and B cells. It has been shown that thymocytes as well as T cells in the spleen become tolerant to, e.g., human gammaglobulin (HGG)[1] in less than 1 day (1). Also, splenic B cells were rapidly tolerized to HGG, the time period being less than 1 day (1) or varying from 2 to 4 days (2). Thus, there appears to be no major differences between splenic T and B cells with regard to tolerance induction, but there is a pronounced difference with regard to the kinetics of the spontaneous loss of tolerance, which occurs in less than 60 days in B cells, but requires more than 100 days in T cells (2).

It has been shown that the polyclonal B-cell activator lipopolysaccharide (LPS) can interfere with induction of tolerance (3) and is able to change an otherwise tolerogenic stimulus into an immunogenic one (4–6). LPS also has the ability to make haptens immunogenic (7). These effects of LPS do not appear to require participation of T cells and are likely to represent a direct effect of LPS on B cells (2, 5).

Polyclonal B-cell activators (PBA) are ligands which directly activate B cells into division and polyclonal immunoglobin secretion without participation of helper cells, such as T cells and macrophages (8). It has been suggested that the triggering signal given by PBA in polyclonal activation is the same as that responsible for induction of specific antibody synthesis after injection of thymus-independent antigens (9) and, consequently, that the immunoglobin receptors do not deliver any triggering signals to B cells (9, 10). It follows from this concept that the immunoglobin receptors also cannot be responsible for the signal delivery in induction of paralysis. Since thymus-dependent (TD) antigens lack the inbuilt triggering property of thymus-independent (TI) antigens (11), TD antigens cannot directly activate B cells. Therefore, tolerance to a TD antigen cannot exist at the B-cell level, if tolerance is defined as a change from the resting state to a tolerant state as a consequence of a signal. A formal proof for the existence of an induced state of tolerant B cells (or the absence of immunocompetent B cells) would be to activate B cells from tolerant animals by PBA and fail to detect induction of antibodies

* This work was supported by grants from the Swedish Cancer Society and the Swedish Medical Research Council.

[1] *Abbreviations used in this paper:* BSS, balanced salt solution; FITC, fluorescein isothiocyanate; HGG, human gamma globulin; LPS, lipopolysaccharide; PBA, polyclonal B-cell activators; PFC, plaque-forming cells; TD, thymus-dependent; TI, thymus-independent.

against the tolerogen. Only one such study has been performed as yet (12) and it was indeed found that cells from animals tolerized by injections of free hapten were activated by PBA to antihapten antibody synthesis.

In the present paper it was studied whether tolerance induced against a TD hapten-protein complex results in deletion or inactivation of the specific B cells, or whether PBA activation could reveal normal numbers of immunocompetent B cells in tolerant animals. It was found that TD antigens could not induce tolerance in the B-cell compartment.

Materials and Methods

Mice. F_1 hybrids between the inbred strains A and B10.5M were used in experiments.

Mitogens. LPS from *Escherichia coli* O55:B5, obtained from Dr. T. Holme, Department of Bacteriology, Karolinska Institute, Stockholm, Sweden, was prepared by phenol-water extraction.

Preparation of Lymphocytes. Spleens were removed and teased with forceps in ice-cold balanced salt solution (BSS). After brief sedimentation, the cells in the supernates were washed three times in 50 ml of cold BSS and subsequently suspended in culture medium to the desired cell concentration. Cellular and viability counts were performed in hemocytometers after staining the damaged cells with 0.02% trypan blue.

Medium. The medium used in most of the experiments was Eagle's minimum essential medium in Earle's solution, supplemented with glutamine, nonessential amino acids, and pyruvate and containing 100 IU of penicillin and 100 μg of streptomycin/ml, as described by Mishell and Dutton (13). The medium was further buffered by 10 mM of HEPES and the pH adjusted to 7.0–7.2. All these reagents were obtained from Flow Laboratories, Irvine, Scotland. All experiments were carried out in serum-free medium (14).

Culture Conditions. Induction of antibody synthesis was always performed in 3-cm diameter plastic Petri dishes (Nunc, Denmark), with a cell concentration of 10^7 spleen cells/ml in 1 ml cultures, set up in triplicate. Cultures were incubated at 37°C in plastic boxes filled with a mixture of 10% CO_2, 83% N_2, and 7% O_2 and were rocked on a platform at 8 oscillations/min.

Assay of Antibody Synthesis. A modified (15) hemolytic plaque assay (16) was used to quantitate cellular antibody production in culture. Cells were harvested with a plastic spatula and washed twice in cold BSS and adjusted to the desired cell concentration. 0.6 ml of 0.5% agar (Bacto Agar; Difco Laboratories, Detroit, Mich.) in BSS containing 0.05% DEAE-dextran (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) was added to 3-ml plastic tubes, which were kept at 46°C in a water bath. Thereafter, 0.05 ml of the indicator erythrocytes diluted 1:8 in BSS, 0.2 ml of the lymphocyte cell suspension, and 0.05 ml of guinea pig serum diluted 1:4 in BSS were added to each tube. The ingredients were mixed and the mixture was evenly spread on plastic Petri dishes with 9-cm diameters, which were thereafter incubated for 3 h at 37°C. Plaque-forming cells (PFC) were counted, using indirect light. Sheep erythrocytes haptenated with different concentrations of the fluorescein isothiocyanate (FITC) hapten were used as indicators in the assay. The sheep erythrocytes, always obtained from the same donor, were stored in sterile Alsever's solution and washed twice in BSS before haptenation. Washed sheep erythrocytes were suspended in a carbonate-bicarbonate buffer, pH 9.2 (20%, vol/vol), and kept with permanent stirring at room temperature. FITC was then added to the erythrocytes and the mixture incubated for 40 min at room temperature. The haptenated erythrocytes were finally washed extensively in BSS and used in the plaque assay. A concentration of 0.5 mg/ml of FITC during conjugation was used to detect high affinity and of 5 mg/ml to detect low affinity anti-FITC PFC.

Conjugation of FITC to Various Carriers. FITC (Isomer 1; BDH Chemicals, Poole, England) was conjugated to proteins according to the method of Bergquist and Schilling (17). In short, FITC was dissolved in bicarbonate buffer, pH 9.23. The FITC concentration was 10 mg/ml. 3.3 ml of the FITC solution were enclosed in dialysis bags. The bags were immersed in 10-ml HGG solutions containing 10 mg protein/ml. The proteins used were previously dissolved in the same bicarbonate buffer, pH 9.23. The mixture was stirred for 24 h at 0°C and thereafter the protein solution was added to a Sephadex G-25 column and eluted with a phosphate buffer, pH 7. The protein solution was filtered until sterile and made up to a protein concentration of 2.5 mg/ml.

LPS could not be efficiently labeled by the above procedure, and therefore the following method was adopted (18). 10 mg/ml of LPS were dissolved in bicarbonate buffer, pH 9.2, and in a separate vessel 2 mg/ml of FITC was dissolved in the same buffer. To 1 ml LPS solution was added 3 ml of the FITC solution. The mixture was stirred overnight and LPS separated from nonreacted FITC by passage through Sephadex G-25 column. The FITC content per mole of protein or LPS was kindly determined spectrophotometrically by Dr. R. Bergquist, National Bacteriological Laboratory, Stockholm, Sweden, according to the method of McKinney et al. (19). The HGG preparation was $FITC_{10}$-HGG and the LPS solution was $FITC_{10}$-LPS. In the latter case a mol wt of LPS of 10^6 was assumed (4 μg FITC/mg LPS).

Preparation of Aggregated FITC-HGG. The $FITC_{10}$-HGG described above was either used in soluble form or precipitated with 9% alum adjusted to pH 6.5. The precipitate was extensively washed, the pH adjusted to 7.2, and it was finally resuspended in BSS at a concentration of 2 mg/ml. It was injected at a dose of 400 μg/mouse together with 10^9 *Bordetella pertussis* bacteria intraperitoneally.

Preparation of Deaggregated FITC-HGG. The procedure of Chiller and Weigle (20) was followed. Thus, $FITC_{10}$-HGG was centrifuged for 2 h at 150,000 *g* and thereafter the upper one-third of the material was removed and immediately injected intravenously in a dose of 2.5 mg/mouse in a total vol of 0.1 ml.

Experimental Procedure. $(A \times 5M)F_1$ mice (eight mice per experiment) were injected with 2.5 mg deaggregated HGG at day 0, followed by a second injection given between 1 and 5 days after the first. After an additional 1–11 days the spleens were removed from three mice and added in culture together with LPS or in some experiments with FITC-LPS. The PFC response against FITC was determined 2 or 4 days later. The five remaining tolerogen-treated animals, as well as five previously untreated mice, were immunized with precipitated FITC-HGG plus *B. pertussis* bacteria. This immunization was repeated and 5 to 10 days later the spleen of the animals were tested for PFC against FITC. In addition, the cells from each individual mouse were added in culture together with LPS and the anti-FITC PFC response determined 2 days later.

This experimental protocol makes it possible to reveal the existence of resting immunocompetent anti-FITC cells in animals within 4–11 days after tolerance induction as well as in animals which in addition have been immunized twice and thereafter tested 13–19 days after tolerance induction. The immunization also constitutes the control for successful tolerance induction.

Results

Spleen Cells from Tolerant Animals are Activated by LPS to Synthesize Antibodies against the Tolerogen. As can be seen in Table I and Fig. 1, spleen cells taken 4–11 days after injection of a total of 5 mg FITC-HGG given in two injections and put in culture for 2 days did not develop spontaneous anti-FITC PFC and were in this respect comparable to untreated controls. However, when given an optimal polyclonal concentration of LPS (100 μg/ml) cells from tolerant animals exhibited a marked increased number of both high and low affinity anti-FITC PFC, which reached the same level as in previously untreated animals when the time period between the last tolerizing dose and the removal of the spleen was 6 days, whereas there was a somewhat suppressed response when the interval was short (1 day). However, also in this case there was a very pronounced increase of the number of anti-FITC PFC response, being about fivefold of the background.

It was also tested whether these spleen cells were specifically tolerant to FITC by trying to induce a specific immune response against the thymus-independent antigen $FITC_{10}$-LPS. As shown in Fig. 2, $FITC_{10}$-LPS caused an immune response in spleen cells from untreated animals, whereas there was no response with cells from tolerant animals, indicating that these cells were indeed tolerant to FITC, even when attempts were made to stimulate a specific immune response by FITC coupled to a thymus-independent carrier.

TABLE I

*In Vitro Induction of Anti-FITC PFC by LPS in Spleen Cells from Animals Injected Twice with Deaggregated FITC-HGG**

Exp. no.	Treatment of mice		In culture		Anti-FITC PFC/10^6 cells	
	With	At days	Day	With	High affinity	Low affinity
1	–			–	2	11
	–			LPS	124	197
	DA‡ FITC-HGG	0 and 5	11	–	6	15
	DA FITC-HGG	0 and 5	11	LPS	68	190
3	–			–	11	26
	–			LPS	234	429
	DA FITC-HGG	0 and 4	5	–	13	28
	DA FITC-HGG	0 and 4	5	LPS	60	123

* Spleen cells from animals treated as described in the Materials and Methods were cultivated in vitro with or without 100 μg LPS for 2 days. Thereafter, three cultures per group were pooled and assayed for the number of anti-FITC PFC.

‡ DA, deaggregated.

Immunization of Tolerant Animals does not Change the Response to LPS. In order to verify the tolerant state of the animals at the time when their cells could be activated by LPS to produce specific anti-FITC PFC, the animals were immunized twice with precipitated FITC-HGG together with *B. pertussis* bacteria. The animals were tested 8–14 days after immunization for direct and, in some experiments, indirect PFC against FITC. In addition, the cells were added in culture in the presence or absence of LPS. As shown in Table II, the tolerant animals failed to mount a specific anti-FITC response after immunization and usually contained the same or lower numbers of PFC as compared to completely unimmunized mice. In contrast, immunized animals exhibited the expected high number of both direct and indirect PFC. Thus the tolerogen-treated animals remained tolerant after immunization.

However, when the same cells were activated by LPS in vitro they developed high numbers of specific anti-FITC PFC, the numbers being equal or nearly equal to those found in previously untreated animals stimulated by LPS (Table III). The longer the interval between the last FITC-HGG injection and addition to culture, the smaller was the difference between untreated and tolerant animals. Thus, spleen cells from tolerant animals could be activated by LPS into synthesis of antibodies against the tolerogen.

Discussion

In these studies tolerance to FITC-HGG was induced by conventional procedures and the tolerant animals or cells from these animals failed to become immunized against a TD and a TI immunogen. The experimental system was selected because it is well analyzed and appears to represent a "classical" tolerance situation without involvement of suppressor T cells (2).

Since polyclonal concentrations of LPS always induced the formation of

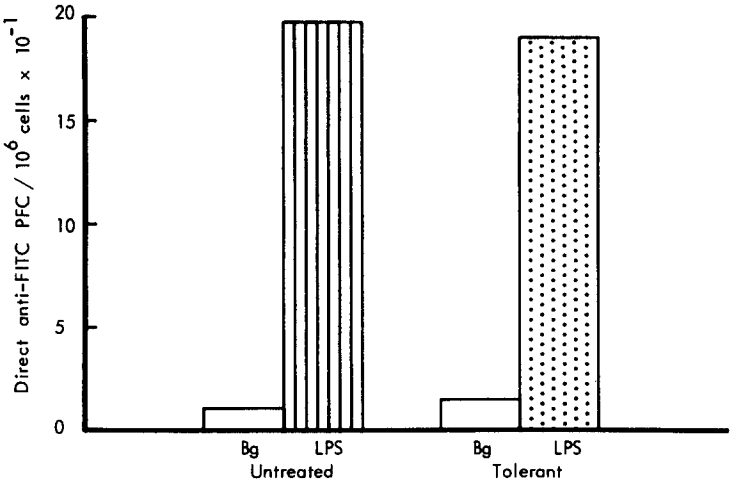

FIG. 1. Effect of LPS on spleen cells from untreated animals or animals tolerized by two injections of deaggregated FITC-HGG (day 0 and 5) and put in serum-free cultures at day 11 with or without LPS. The anti-FITC response was determined 2 days later. Open bars represent background (Bg) response in untreated serum-free cultures, and striped and dotted bars represent the response to 100 μg LPS.

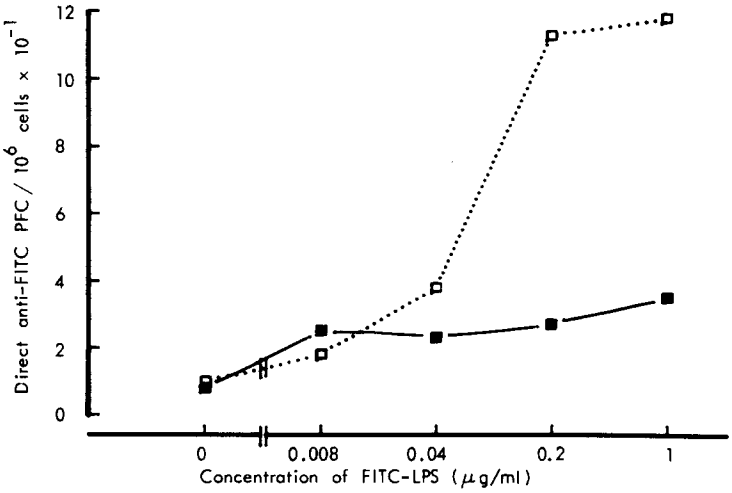

FIG. 2. The same spleen cells as in Fig. 1, but activated in serum-free culture with the indicated concentrations of $FITC_{10}$-LPS and the response to FITC determined after 4 days in culture. (□---□), spleen cells from untreated mice; (■—■), spleen cells from tolerant animals.

antibodies against the tolerogen it must be concluded that specific B cells in tolerant animals are present in normal numbers. Thus, tolerance cannot be due to a process leading to elimination or functional inactivation of specific B cells. Since these B cells failed to become specifically activated by TI (FITC-LPS) or TD (FITC-HGG) antigens, the most likely explanation of the findings is that the Ig receptors were incapable of binding the antigenic determinants.

We suggest that the most simple explanation of the presence of B cells that

TABLE II

Immune Response of Tolerant and Untreated Mice after Immunization with Aggregated FITC-HGG

Exp. no.	Tolerogenic		Immunogenic		Assayed on day	Log_{10} PFC/10^6 cells*			
						Direct		Indirect	
	Treatment	At days	Treatment	At days		High affinity	Low affinity	High affinity	Low affinity
1	–		–						1.35 (22)
	–		A‡ FITC-HGG	11 and 13	19				2.33 ± 0.17 (220)
	DA FITC-HGG	0 and 5	A FITC-HGG	11 and 13	19				0.30 ± 0.3 (2)
2	–		–			Neg§	0.79 (6)		Neg
	–		A FITC-HGG	4 and 8	13	1.51 ± 0.3 (32)	2.52 ± 0.07 (331)		2.73 ± 0.09 (537)
	DA FITC-HGG	0 and 1	A FITC-HGG	4 and 8	13	Neg	Neg		1.26 ± 0.2 (18)
3	–		–	5 and 10		0.18 (15)	1.57 (37)	Neg	1.18 (15)
	–		+ FITC-HGG	5 and 10	19	1.92 ± 0.08 (83)	2.44 ± 0.07 (275)	2.15 ± 0.14 (141)	2.61 ± 0.21 (407)
	DA FITC-HGG	0 and 4	A FITC-HGG		19	Neg	Neg	1.28 ± 0.17 (19)	1.33 ± 0.15 (21)

* Log_{10} mean ± SE anti-log within parentheses.

‡ A, aggregated; and DA, deaggregated.

§ Neg, a mean of less than 1 PFC/10^6 cells.

TABLE III

Activation by LPS of spleen cells from tolerant animals being immunized with aggregated FITC-HGG

Exp. no.	Tolerogenic		Immunogenic		In culture		Anti-FITC PFC/10^6			
							Direct		Indirect	
	Treatment	At days	Treatment	At days	Day	With	High affinity	Low affinity	High affinity	Low affinity
1	–		–			–	13	35	0	0
	–		–			LPS	165 ± 10	372 ± 12	0	0
	DA* FITC-HGG	0 and 5	A FITC-HGG	11 and 13	19	–	4 ± 2	4 ± 1	0	0
	DA FITC-HGG	0 and 5	A FITC-HGG	11 and 13	19	LPS	101 ± 22	218 ± 55	0 ± 1	0
	–		A FITC-HGG	11 and 13	19	–	16 ± 4	52 ± 2	17 ± 2	20 ± 3
	–		A FITC-HGG	11 and 13	19	LPS	155 ± 24	365 ± 78	43 ± 7	153 ± 24
2	–		–			–	12 ± 7	11 ± 1		
	–		–			LPS	45 ± 5	340 ± 12		
	DA FITC-HGG	0 and 1	A FITC-HGG	4 and 8	13	–	5 ± 1	9 ± 3		
	DA FITC-HGG	0 and 1	A FITC-HGG	4 and 8	13	LPS	56 ± 5	251 ± 15		
	–		A FITC-HGG	4 and 8	13	–	80 ± 23	158 ± 21		
	–		A FITC-HGG	4 and 8	13	LPS	314 ± 110	839 ± 61		
3	–		–			–	14	35		
	–		–			LPS	111	459		
	DA FITC-HGG	0 and 4	A FITC-HGG	5 and 10	19	–	4 ± 2	13 ± 4		
	DA FITC-HGG	0 and 4	A FITC-HGG	5 and 10	19	LPS	82 ± 7	235 ± 40		
	–		A FITC-HGG	5 and 10	19	–	39 ± 8	76 ± 13		
	–		A FITC-HGG	5 and 10	19	LPS	173 ± 11	436 ± 35		

* DA, deaggregated; and A, aggregated.

can be activated by PBA but not by antigen, is that the Ig receptors are blocked by the tolerogen. This has not been directly studied in these or previous experiments, but the findings of Aldo-Benson and Borel (21) have clearly demonstrated antigen on B cells from tolerant animals. A number of other findings are also compatible with this possibility. Thus, tolerance to certain haptens (22) and to LPS (23) is rapidly broken if the cells are cultured in vitro in the absence of the tolerogen or by simply washing the cells before addition to the cultures. It has also been observed (24) that DNP-D-Gly,Lys which is a potent tolerogen, actually binds to about 3% of the spleen cells in vitro and remains attached to the cell surface during in vitro cultivation.

The findings that tolerance to TD antigens could be broken by the injection of cross-reacting antigens was already earlier explained on the basis that the tolerant animals possessed responsive B cells but lacked specific immunocompetent T cells (25). The present findings go one step further by postulating that there is always a normal complement of B cells in tolerant animals irrespective of the time after tolerance induction and thus, that there exists no true B-cell tolerance to TD antigens. However, it does not necessarily follow from this that it should be possible to break tolerance by the injection of cross-reacting antigens at any time period after tolerance induction, since the reason for the inability to trigger these cells with a hapten TI carrier is presumably that the Ig receptors are blocked by antigen. In such a case it is not to be expected that injection of cross-reacting antigen, causing activation of new T cells, would be competent to break tolerance, since the responding B cells have their receptor occupied by antigen. It is not known how long the receptor blockade could persist, but the finding that tolerance can be broken by cross-reacting antigen suggests that it does not persist for very long time periods. Therefore, the spontaneous appearance of immune responsiveness as determined by, e.g. cell reconstitution experiments or injection of cross-reacting antigens may in fact be a measure of the time it takes to clear the Ig receptors from antigen, rather than the time necessary for the reappearance of antigen-reactive B cells.

It was observed that when the interval between the tolerogenic or immunogenic challenge and the addition of cells from the treated animals to culture was short, there was a less than normal LPS-induced response to the hapten, whereas there was a normal response when the interval was longer (11 days). This cannot have been due to partial tolerance at the B-cell level, since the animals were completely tolerant, both when attempts were made to immunize them in vivo and in vitro with the hapten or a TI carrier. Most likely this is due to some trivial effects, possibly caused by the fact that the antigen is an immunoglobulin as pointed out before (2) or else caused by a phenomenon analogous to effector cell blockade (26), in which antigen bound to the Ig receptor cause some disturbance of the cell, which is expressed as an inability to secrete antibodies. Whatever the mechanism, the small suppression observed here with cells from tolerant animals is most likely due to a nonspecific effect on the cell surface not relevant for the triggering process.

Our findings show that the only stringent test for the presence of tolerant B cells in tolerant animals is the failure of PBA to activate antibody synthesis against the tolerogen. This test has now been used in two different test systems

and in both cases were immunocompetent B cells found in tolerant animals (12). Analogous studies have not as yet been performed by others. It must be concluded that TD antigens cannot induce tolerance in B cells, but only in T cells. This is in accordance with the postulates of the one nonspecific signal hypothesis (8, 9), but is incompatible with a two signal hypothesis, which is based on signal discrimination for the induction of tolerance and immunity. Another consequence of the one nonspecific signal concept as well as from the present findings, is that immunocompetent B cells towards TD autoantigens should normally exist. However, they cannot normally be activated into antibody synthesis, because there are no helper T cells available.

Summary

Immunological tolerance was induced in adult mice by the injection of 5 mg of deaggregated hapten-protein conjugate. The tolerant state was confirmed 4–19 days later by the failure of such animals to mount an immune response against an aggregated form of the same thymus-dependent hapten-protein conjugate as well as by the inability of spleen cells from tolerant animals to respond to a thymus-independent hapten-carrier conjugate. Even though the animals were fully tolerant, their spleen cells were activated by lipopolysaccharide (LPS) in vitro to produce normal numbers of plaque-forming cells against the hapten.

The finding that spleen cells from tolerant animals could be activated by LPS into synthesis of antibodies against the tolerogen indicates that tolerance to thymus-dependent antigens does not affect B cells, but presumably only T cells. It is suggested that the only stringent test for the existence of B-cell tolerance is the inability of polyclonal B-cell activators to activate antibody synthesis against the tolerogen. The findings make it unlikely that B-cell tolerance to autologous thymus-dependent antigens exists and further indicate that such antigens cannot deliver activating or tolerogenic signals to B cells, although they are competent to combine with and block the Ig receptors.

The technical assistance of Miss Inger Cederberg and Miss Yrsa Avellan is gratefully acknowledged.

Received for publication 16 December 1975.

References

1. Chiller, J. M., and W. O. Weigle. 1973. Restoration of immunocompetence in tolerant lymphoid cell populations by cellular supplementation. *J. Immunol.* **110**:1051.
2. Chiller, J. M., J. A. Louis, B. J. Skidmore, and W. O. Weigle. 1974. Cellular parameters of the tolerant state induced to human γ-globulin in mice and its modulation by bacterial lipopolysaccharide. *In* Immunological Tolerance. D. H. Katz and B. Benacerraf, editors. Academic Press Inc., New York. 373.
3. Golub, E. S., and W. O. Weigle. 1967. Studies on the induction of immunological unresponsiveness. I. Effects of endotoxin and phytohemagglutinin. *J. Immunol.* **98**:1241.
4. Claman, H. N. 1963. Tolerance to a protein antigen in adult mice and the effect of nonspecific factors. *J. Immunol.* **91**:833.
5. Louis, J. A., J. M. Chiller, and W. O. Weigle. 1973. The ability of bacterial lipopoly-

saccharide to modulate the induction of unresponsiveness to a state of immunity. *J. Exp. Med.* 138:1481.

6. Oruellas, E. P., F. Sanfilippo, and D. W. Scott. 1974. Cellular events in tolerance. IV. The effect of a graft-versus-host reaction and endotoxin on hapten- and carrier-specific tolerance. *Eur. J. Immunol.* 4:587.
7. Schmidtke, J. R., and F. J. Dixon. 1972. Immune response to a hapten coupled to a nonimmunogenic carrier. Influence of lipopolysaccharide. *J. Exp. Med.* 136:392.
8. Coutinho, A., and G. Möller. 1975. Thymus-independent B cell induction and paralysis. *Adv. Immunol.* 21:114.
9. Coutinho, A., and G. Möller. 1974. Immune activation of B cells: evidence for "one non-specific triggering signal" not delivered by the Ig receptors. *Scand. J. Immunol.* 3:133.
10. Coutinho, A., and G. Möller. 1975 The self-nonself discrimination. A one signal mechanism. *Scand. J. Immunol.* 4:99.
11. Coutinho, A., and G. Möller. 1973. B cell mitogenic properties of thymus-independent antigen. *Nat. New Biol.* 245:12.
12. Gronowicz, E., and A. Coutinho. 1975. Hapten-induced B cell paralysis. II. Evidence for trivial mechanisms of tolerance. *Eur. J. Immunol.* 5:413.
13. Mishell, R., and R. W. Dutton. 1966. Immunization of normal mouse spleen cell suspensions in vitro. *Science (Wash. D. C.)* 153:1004.
14. Coutinho, A., and G. Möller. 1973. In vitro induction of specific immune responses in the absence of serum: requirement for non-specific T or B cell mitogens. *Eur. J. Immunol.* 3:531.
15. Bullock, W. W., and E. Möller. 1972. "Spontaneous" B cell activation due to loss of normal mouse serum suppressors. *Eur. J. Immunol.* 2:514.
16. Jerne, N. K., and A. A. Nordin. 1965. Plaque-formation in agar by single antibody producing cells. *Science (Wash. D. C.).* 140:405.
17. Bergquist, N. R., and W. G. E. E. Schilling. 1970. Preparation of anti-human immunoglobulin for direct fluorescent tracing of autoantibodies. *In* Standardization in Immunofluorescence. E. J. Holborow, editor. Blackwell Scientific Publications Ltd., Oxford, England. 171.
18. Möller, G., A. Coutinho, and U. Persson. 1975. Mechanism of B lymphocyte activation: failure to obtain evidence of a direct role of the Ig receptors in the triggering process. *Scand. J. Immunol.* 4:37.
19. McKinney, R. M., J. T. Spillane, and G. W. Pearce. 1964. Fluorescein diacetate as a reference colour standard in fluorescent antibody studies. *Anal. Biochem.* 9:474.
20. Chiller, J. M., and W. O. Weigle. 1971. Cellular events during induction of immunological unresponsiveness in adult mice. *J. Immunol.* 106:1647.
21. Aldo-Benson, M., and Y. Borel. 1974. The tolerant cell: direct evidence for receptor blockade by tolerogen. *J. Immunol.* 112:1793.
22. Gronowicz, E., A. Coutinho, and O. Sjöberg. 1974. Hapten-induced paralysis I. Evidence for the persistence of hapten-specific immunocompetent B lymphocytes in specifically paralyzed mice. *Eur. J. Immunol.* 4:226.
23. Sjöberg, O. 1972. Rapid breaking of tolerance against *Escherichia coli* lipopolysaccharide in vivo and in vitro. *J. Exp. Med.* 135:850.
24. Ault, K. A., E. R. Unanue, D. H. Katz, and B. Benacerraf. 1974. Failure of lymphocytes to re-express antigen receptors after brief interaction with a tolerogenic D-amino acid copolymer. *Proc. Natl. Acad. Sci. U. S. A.* 71:3111.
25. Weigle, W. O., J. M. Chiller, and G. S. Habicht. 1972. Effect of immunological unresponsiveness and different cell populations. *Transplant. Rev.* 8:3.
26. Schrader, J. W., and G. J. V. Nossal. 1974. Effector cell blockade. A new mechanism of immune hyporeactivity induced by multivalent antigen. *J. Exp. Med.* 139:1582.