

ANTIGEN-BINDING CELLS IN MICE IMMUNE OR TOLERANT TO
ESCHERICHIA COLI POLYSACCHARIDE*

BY OLOF SJÖBERG, M.D.

(From the Division of Immunobiology, Karolinska Institutet, Wallenberglaboratoriet,
104 05 Stockholm 50, Sweden)

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The mechanism of immunological tolerance (for review see ref. 1) is still largely unknown. Thus, it is not known whether tolerance is caused by inactivation or by killing of the immunocompetent cells. In the course of the normal immune response antibody-secreting cells, as well as antigen-binding cells, can be detected. Antibody-secreting cells can be enumerated *in vitro* by the technique of local hemolysis in gel (2) and are generally called plaque-forming cells (PFC).¹ Antigen-binding cells may be visualized by their ability to bind particulate antigens, such as sheep red cells (SRC), to their surfaces and these cells have been termed rosette-forming cells (RFC) (3, 4). It is likely that RFC represent a mixture of antibody-secreting and nonsecreting cells (5).

Howard et al. (6) studied the RFC response in the spleens of mice tolerant to pneumococcal polysaccharide. Although serum antibodies could not be detected in these animals, a considerable number of RFC was found, which in fact was larger in the "tolerant" than in the optimally immunized animals. They suggested that antibody-secreting cells were present in these animals but the humoral antibodies produced were neutralized extracellularly by persisting undegraded antigen. To test if this hypothesis was true for another polysaccharide, the presence of antigen-binding cells was examined in mice tolerant towards polysaccharide of *Escherichia coli* origin as judged by the absence of antibody production at the cellular level.

It has recently been shown that a significant proportion of RFC against sheep red blood cells are thymus-derived lymphocytes (T-cells) (5). SRC are known to be dependent on the presence of T-cells for induction of an optimal antibody response (for reviews see ref. 7). Recently it has been shown that coli polysaccharide is a nonthymus-dependent antigen.² Therefore, experiments

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¹ *Abbreviations used in this paper:* B-cells, bone marrow-derived lymphocytes; BSS, balanced salt solution; CPS, coli polysaccharide; CPS-MRC, CPS-sensitized CBA mouse red cells; PFC, plaque-forming cells; RFC, rosette-forming cells; SRC, sheep red cells; T-cells, thymus-derived lymphocytes.

² Möller, G., and G. Michael. To be published.

were performed to study the existence of RFC of T-cell origin also against such a nonthymus-dependent antigen.

Materials and Methods

Mice.—Inbred CBA mice 2–5 months old were used.

Extraction and Detoxification of Coli Polysaccharide (CPS).—Lipopolysaccharide (CPS) of *E. coli* 055:B5 was obtained by phenol extraction according to the method of Westphal, et al. (8) with some modifications (9).

Detoxification was performed with weak alkali as described by Neter et al. (10). The detoxified material was kept as a dry substance at 4°C and was dissolved in saline immediately before use.

Immunizations.—For an optimal antibody response 0.01 mg of CPS was injected intraperitoneally in 0.2 ml of balanced salt solution (BSS) (11). SRC kept in Alsever's solution were washed once in BSS before immunization and 4×10^8 SRC suspended in BSS were injected intraperitoneally.

Tests for PFC and RFC were made 5 days after the injection of the relevant antigen if not otherwise stated.

Tolerance Induction.—Tolerance was induced by five injections of 3 mg of alkali detoxified CPS during 14 days and maintained by weekly injections of 3 mg of CPS. Routinely the tolerant mice were given a test dose of 0.01 mg of CPS 7 days after the last tolerance-maintaining dose and assayed 5 days later for PFC and RFC.

Local Hemolysis in Gel Assay.—The number of PFC was assayed according to Jerne and Nordin (2) as modified by Mishell and Dutton (12). The spleens were pressed through a 60-mesh stainless steel screen into BSS. Cell clumps were removed by filtration through gauze, the cells were washed three times in BSS, and suspended to the appropriate cell concentration in BSS. The number of viable cells was estimated with the trypan blue-exclusion technique. 0.1 ml of the cell suspension and 0.025 ml of the relevant red blood cells diluted 1:7.5 in BSS were added to 0.5 ml of a 0.5% agarose solution in glass tubes kept at 45°C. The mixture was poured onto microscope glass slides. Duplicate slides were made for each cell suspension. The slides were put on racks and incubated at 37°C with guinea pig complement diluted 1:20 in BSS. Plaques were read without staining in indirect light using a magnifying glass.

Cellular antibody synthesis to CPS was studied by coating SRC with CPS according to Möller (13). To calculate the number of PFC directed against CPS, the number of PFC against untreated SRC was subtracted from the number of PFC against CPS-sensitized SRC obtained with the same cell suspension. Since CPS stimulates 19S antibody synthesis exclusively for a prolonged period, only direct PFC were studied (14).

RFC Assay.—The number of RFC was assayed by a slight modification of the centrifugation-suspension technique of McConnell et al. (15). The spleen cells, which had been washed three times, were adjusted to a cell density of 10×10^6 cells/ml in calcium-free BSS. 0.5 ml of this cell suspension was mixed with 0.5 ml of a suspension of CPS-sensitized CBA mouse red cells (CPS-MRC) containing 50×10^6 red cells/ml in a small tube. Accordingly, the proportion of spleen cells to red cells was 1:5. The CPS was absorbed onto CBA mouse erythrocytes with the technique used for sensitization of SRC described above. The mixture of spleen cells and CPS-MRC was spun at 100 *g* for 10 min. The cells were suspended by gently turning the tube repeatedly, and the number of RFC was determined by counting in a hemocytometer. Cells with five or more red cells adhering were regarded as RFC. 45,000 cells of each cell suspension were scored. The cells were kept at +4°C during the assay.

Anti-Theta Serum Treatment.—Anti-theta serum was raised in adult AKR mice by immunization with CBA thymocytes intraperitoneally and subcutaneously. The injections were

given once a week during 4 wk and thereafter once every 14 days. Serum was collected 6 days after the last immunization. 100% of thymocytes, 20–40% of spleen cells, and 0% of bone marrow cells were killed by the serum. The cytotoxic titer of the serum against CBA thymus cells was 1:64–1:128. Absorption with bone marrow cells had no effect whereas absorption with thymocytes or brain cells completely abolished the cytotoxicity against thymocytes. Further specificity tests have been performed by Greaves and Möller (5). Normal AKR serum was used as control serum. Treatment of spleen cells with anti-theta serum was performed *in vitro*. Approximately 100×10^6 washed spleen cells were treated with 0.3 ml of undiluted anti-theta serum for 30 min at 37°C. Thereafter the spleen cells were washed once and 0.3 ml of a 1:6 dilution of lyophilized guinea pig complement was added to the cells and the incubation continued for 30 min at 37°C. Finally, the cells were washed and resuspended in BSS to the appropriate concentration.

Anti-Immunoglobulin Serum.—The rabbit anti-mouse immunoglobulin serum was prepared as described by Möller (22). The serum was absorbed with CPS-coated MRC to remove antibodies against CPS and MRC.

RESULTS

The PFC and RFC Responses against CPS.—Spleen cells from normal animals or animals immune or tolerant against CPS were tested for their numbers of PFC and RFC (Table I). In immune mice the assay was done 5 days after an injection of 0.01 mg of CPS at the peak of the PFC response (11). In one of the tolerant groups (tolerant mice A), the assay was done 5 days after the last injection of the tolerance-maintaining dose of 3 mg of CPS. In the other tolerant group (tolerant mice B), the animals received an injection of 0.01 mg and were tested 5 days later. Although the number of PFC increased from 0.5 PFC/ 10^6 spleen cells in normal animals to 209 PFC in optimally immunized animals, there was only a fivefold increase of the number of RFC after an optimal immunization. In animals tolerant to CPS, as judged by no or only a minimal PFC response, no decrease of the number of RFC was found as compared to the number in normal mice. In fact the number of RFC was slightly increased in the group tolerant mice A ($P < 0.05$) and highly significantly increased in the group tolerant mice B ($P < 0.001$). However, the number of RFC was not as high in tolerant mice as in immune mice.

Specificity of RFC against CPS.—The presence of RFC against autologous erythrocytes both in thymus and in spleen has been observed (Micklem, personal communication). To test whether such a phenomenon could explain the RFC found in tolerant animals, the number of RFC against untreated CBA erythrocytes was tested in mice unprimed, primed, or tolerant against CPS (Table II). Only approximately 30 RFC/ 10^6 spleen cells were found in normal and tolerant animals. In mice given 0.01 mg of CPS and assayed for auto-RFC 5 days later, the number of RFC was 58/ 10^6 spleen cells. This number is not significantly different from that in normal and tolerant animals. This is in contrast to the results of Micklem, which indicated an increase of the number of auto-RFC in animals after an immunization. However, due to

the low RFC counts obtained in the present experiments, the variation is quite pronounced.

If the RFC against CPS-MRC are specific for CPS, it should be possible to inhibit them by pretreating the spleen cells with free CPS before adding the sensitized MRC. Table III shows that such treatment inhibited the formation of RFC completely or almost completely.

TABLE I
Cellular Immune Response against CPS in Spleens from Normal, Immune, and Tolerant Mice

Mice	PFC/10 ⁶ spleen cells	RFC/10 ⁶ spleen cells	No. of mice
Normal	-0.30 ± 0.15 (0.5)*	1.97 ± 0.03 (93)	9
Immune†	2.32 ± 0.09 (209)	2.71 ± 0.07 (513)	10
Tolerant A§	-0.23 ± 0.22 (0.6)	2.21 ± 0.14 (164)	5
Tolerant B	0.34 ± 0.22 (2.2)	2.38 ± 0.03 (240)	10

* The figures are given in ₁₀logs for the mean ± standard error. The corresponding antilogs for the mean are given in parenthesis.

† Tested 5 days after an injection of 0.01 mg of CPS.

§ Tested 5 days after the last tolerance-maintaining dose of 3 mg of CPS.

|| Tolerant mice B were given 0.01 mg of CPS 7 days after the last injection of 3 mg of CPS and their spleens were assayed 5 days later.

TABLE II
Number of RFC against Autologous Red Blood Cells

Mice	RFC/10 ⁶ spleen cells	No. of mice
Normal	1.46 ± 0.09 (28.8)*	7
Immune†	1.54 ± 0.09 (35.0)	5
Tolerant§	1.76 ± 0.12 (57.7)	5

* ₁₀logs for the mean ± standard error. Antilogs for mean in parenthesis.

† Tested 5 days after an injection of 0.01 mg of CPS.

§ Mice tolerant to CPS were given 0.01 mg of CPS 7 days after the last tolerance-maintaining dose and assayed 5 days later.

Lipopolysaccharides are known to be "sticky" and tend to adhere to cells passively. The conjugation method used in this study depends on such a reaction. It seems possible that also the CPS-sensitized MRC become sticky and therefore form nonspecific rosettes. To test this possibility the spleen cells were pretreated with rabbit anti-mouse immunoglobulin for 30 min since the specific receptors for antigens are considered to be immunoglobulin-like molecules (for reviews see ref. 16). As shown in Table III no or only a few RFC against CPS-RFC were found after such a treatment. Thus it seems likely that the RFC against CPS-MRC represents a specific binding of the CPS-determinants to the immunoglobulin receptors of the spleen cells.

Characteristics of Antigen-Binding Cells in Tolerant and Immune Mice.— It could be argued that the large doses of CPS used to induce tolerance cause a nonspecific toxic depression of the PFC response without affecting the development of RFC to a similar extent, which could explain the RFC found in tolerant animals. The results given in Table IV show that this was not the case. Mice tolerant to CPS gave a normal PFC response to SRC 5 days after an injection of 4×10^8 SRC.

TABLE III
*Number of RFC against CPS after Preincubation of Spleen Cells with Free CPS or Anti-Immunoglobulin Serum**

Mice‡	Preincubation with	
	CPS	Anti-Ig
Normal	22, 0, 22§	0, 22, 44
Immune	44, 0, 22	89, 67, 44, 44
Tolerant	44, 0, 22	22, 14, 44, 22, 22, 0

* The spleen cells were incubated either with free CPS at a concentration of 1 mg/ml or anti-immunoglobulin serum at a dilution of 1/20 for 30 min at +4°C before mixing with CPS-sensitized SRC.

‡ Designations of mice as in Table II.

§ Each figure represents one experiment, in which one to three spleens were pooled.

TABLE IV
Specificity of Tolerance to CPS

Mice	PFC/10 ⁶ spleen cells*	No. of mice
Normal	2.76 ± 0.07 (582)‡	5
Tolerant	2.88 ± 0.03 (757)	5

* The number of PFC against SRC was assayed in spleens from normal mice or mice tolerant to CPS 5 days after an intraperitoneal injection of 4×10^8 SRC.

‡ PFC numbers are given in ₁₀logs for the mean \pm standard error; the corresponding anti-logs are given in parenthesis.

Another possibility is that the RFC in tolerant mice are cells which produce antibodies directed against other determinants than those antibodies causing plaques in optimally immunized animals. It might be speculated that these determinants occur in a very low frequency and therefore can cause formation of rosettes but not of plaques. This implies that rosette formation is less dependent on a high density of the antigenic determinants on the erythrocytes than is the plaque formation. This was tested in an indirect way (Table V). Mice were immunized against SRC and their spleen cells assayed for PFC and RFC against both untreated SRC and CPS-sensitized SRC. It can be

assumed that some of the antigenic determinants specific for the SRC were covered by the CPS at the coating. No inhibition of the PFC was found when CPS-SRC were used instead of ordinary SRC. On the other hand, there was a depression of 40% of the RFC when tested against CPS-RFC. Thus, it seems likely that the above assumption was not true. On the contrary the formation of rosettes seemed to be more dependent on a high density of the antigenic determinants than the formation of plaques.

TABLE V
Per Cent Inhibition of PFC and RFC against SRC after Conjugation of SRC with CPS

Experiment No.	PFC	RFC
1	15.1	53.5
2	-9.5	27.4
3	0.9	31.4
4	-7.6	47.1
Mean	-0.3 ± 5.6	39.9 ± 6.2

Mice were immunized with SRC 5 days before the test. The numbers of PFC and RFC against untreated and CPS-sensitized SRC were compared and the per cent inhibition, when CPS-sensitized SRC were used, was calculated.

TABLE VI
Per Cent Inhibition of PFC and RFC after Treatment with Anti-Theta Serum

Mice	PFC	RFC	No. of mice
Immune against CPS*	2.8 ± 2.9	-0.5 ± 4.1	4
Tolerant against CPS*	N.C.†	-1.0 ± 7.1	4
Immune against SRC§	-3.0 ± 2.7	31.0 ± 1.0	5

* Mice immune or tolerant against CPS were treated as in Table II and assayed against CPS.

† N.C., not calculated.

§ Tested against SRC 5 days after an optimal immunization with SRC.

Both bone marrow-derived (B-cells) and thymus-derived (T-cells) lymphocytes can give rise to RFC against certain antigens (5). If tolerance induction inactivates B-cells preferentially, leaving T-cells relatively unaffected, the increased number of RFC in tolerant animals could be of T-cell origin. To test if this alternative is true, spleen cells from tolerant animals were assayed for RFC and PFC after treatment with anti-theta serum (Table VI). No inhibition of the RFC was obtained. Furthermore, when mice optimally immunized against CPS were tested for the presence of thymus-derived RFC in the same way, no such RFC were detected. In contrast to this, 31% of the RFC against SRC in spleens from mice immunized against SRC could be inhibited by anti-

theta serum treatment. Thus, CPS, in contrast to SRC, will not stimulate T-cells to a RFC response.

The effect of anti-theta serum on PFC was also tested. There was no decrease of the number of PFC, neither against CPS nor against SRC.

DISCUSSION

Thus it was found that mice tolerant to CPS, as judged by the absence of an antibody response, still can respond with an increased number of antigen-binding cells against this antigen. The antigen-binding cells (= RFC) in these animals were found to be specific against CPS. Only very few rosettes were obtained when mixing spleen cells with autologous red cells. The formation of rosettes could be inhibited completely or almost completely by pretreatment with free CPS or rabbit anti-mouse immunoglobulin serum. The inhibition by rabbit anti-mouse immunoglobulin serum shows that the binding of the CPS-sensitized MRC to the spleen cells is not due to a nonspecific adherence caused by the stickiness of the CPS, but to a binding to receptors which are immunoglobulins or shear antigenic determinants with immunoglobulins.

The present findings cannot be explained by postulating that antibodies secreted by antibody-producing cells in tolerant mice are neutralized extracellularly by excess antigen, as was suggested by Howard et al. (6), since antibody production was measured at the cellular level. Thus, tolerance to CPS appears to inhibit selectively the steps leading to the appearance of antibody-secreting cells without affecting those resulting in the development of RFC to a similar extent. In fact, Howard has recently reinvestigated the immune status in mice tolerant to pneumococcal polysaccharide at the cellular level and has also found antigen-binding cells in situations where the antibody-secreting cells are paralyzed by very high antigen doses (personal communication).

The finding of antigen-binding cells in tolerant animals is not unique. It has been reported earlier by Howard et al. (6) as mentioned above, by Ada (17), and by Naor and Sulitzeanu (18). In contrast to the present results, neither Ada nor Naor and Sulitzeanu found any increase of antigen-binding cells over the number found in normal animals. This discrepancy might depend on differences in the properties of the antigen used, in the scheme for tolerance induction, and in the methods for detection of antigen-binding cells.

A number of different alternatives seem possible to explain the finding of an increased number of RFC in tolerant animals. (a) The receptors on the RFC in tolerant animals may be directed against other antigenic determinants than are the antibodies secreted by the PFC in optimally immunized animals. In terms of this possibility it can be assumed that the antigenic determinants occur in such a low density on the CPS-sensitized SRC that antibodies against them do not cause plaques. This possibility seems unlikely because of the

present findings that the formation of rosettes is more dependent on a high density of the antigenic determinants than the formation of plaques. Furthermore, Humphrey and Dourmashkin (19) found that one or two 19S antibody molecules attached to a red cell will cause lysis of the cell. Finally, it is unlikely that the RFC present in tolerant animals are directed against other determinants than are the antibodies causing plaques, also because of recent results of Möller et al. (to be published). They studied the cellular response in mice tolerant against a hapten (NNP) and found a marked decrease of the number of PFC together with a moderate depression of the RFC response as compared with the responses in immune mice, which is in agreement with the present results.

(b) Tolerant animals may contain cells actually secreting antibodies directed against the same determinants as the antibodies producing plaques in immune animals. These antibodies may be of very low affinity, however, in concordance with earlier findings, that tolerance induction affects preferentially the production of high affinity antibodies (20). It would be possible that cells producing antibodies of a very low affinity are detected as RFC but not as PFC. This explanation seems unlikely, however, if one considers that the receptors on the RFC must have an affinity sufficiently high to bind the antigen during the suspension of the cell pellet. It is assumed in this discussion that the antibodies produced have the same properties as the receptors on the precursor cells for antibody production (21). Furthermore, the finding of Möller (22) that 50–100% of a spleen cell population may occasionally be detected as PFC suggests that most of the antibody-secreting cells are, in fact, detected as PFC.

(c) Tolerance induction leads to blockage of antibody secretion without affecting division and differentiation of precursor cells for antibody production. It can be argued against this possibility that RFC are fewer in tolerant than in optimally immunized animals. This would not be the case if tolerance means simply inhibition of secretion of antibodies.

(d) Tolerance may be caused by the inhibition of division and differentiation of antigen-sensitive cells. The RFC in tolerant animals may in that case be formed by bone marrow-derived lymphocytes (B-cells), which do not give rise to PFC or by thymus-derived lymphocytes (T-cells). Recent findings have shown that T-cells and B-cells cooperate in the antibody response to certain antigens (for reviews see ref. 7). It has also been found that a high proportion of the RFC appearing in mice immune to SRC are T-cells, using experimental systems involving isoantigenic markers (H-2, theta) on T-cells (5). The coli polysaccharide is not dependent on T-cells in order to induce a humoral antibody response.² This does not necessarily imply that there are no receptors for the antigen among the T-cells. The independence of T-cells for an adequate immune response to CPS may be related to the fact that the structure of this

antigen is characterized by repeating units of identical determinants. Such an antigen may bind well to bone marrow precursor cells with multivalent receptors and stimulate them to antibody production without the presence of T-cells. Thus, it would be possible that T-cells are stimulated to respond in tolerant animals. Such cells would be revealed as RFC but not as PFC and may therefore be the explanation for the finding of RFC in tolerant animals. However, as shown above there was no inhibition of RFC in tolerant animals against CPS after treatment with anti-theta serum. The RFC against CPS are therefore not likely to be T-cells but rather B-cells.

It is possible that a high dose of antigen will only stop division of B-cells with receptors for the antigen above a certain level of affinity. Such cells would give rise to PFC after an optimal immunization dose. B-cells with receptors of lower affinity would be stimulated to divide but are not driven through all the stages of division and differentiation to antibody production after an optimal immunization dose because the energy of binding of the antigen to the receptors would be too low. These cells are not prevented to divide in tolerant animals since they escape tolerance due to the low affinity of their receptors. Such cells with rather low affinity receptors could therefore be the explanation for the RFC in tolerant animals. If this alternative is correct it would imply that a level of affinity sufficiently high to bind the antigen to the cell surface is not high enough to induce antibody production. However, from these experiments it cannot be excluded that at least some of the RFC in tolerant mice represents true tolerant cells, i.e., cells that after an optimal stimulation would have given rise to antibody production but have become inactivated by the large antigen doses. This would mean that tolerance is caused by inactivation instead of killing of immunocompetent precursor cells for antibody production.

Even after an optimal immunization no theta-positive RFC against CPS could be detected. Because of the low number of RFC against CPS in normal mice, and the uncertainty connected with an assay of the percentage of theta-positive RFC at such low values, no attempt was made to determine the presence of RFC of thymic origin in normal mice. Therefore it cannot be decided at this moment whether the lack of theta-positive RFC is due to a lack of receptors for the antigen among the T-cell population or tolerance induction or lack of stimulation of immunocompetent T-cells.

However, the absence of theta-positive RFC against CPS can explain some of the features of the immune response against this antigen. The inability of T-cells to increase the response follows logically since there are no RFC of thymic origin to cooperate with for the B-cells. Low zone tolerance has not been possible to obtain to CPS (11) or to pneumococcal polysaccharide (23) which both are thymus-independent antigens (5, 24). This is to be expected if low zone tolerance means inactivation of T-cells, as suggested by Mitchison and Rajewski (personal communication). It follows from this reasoning that

low zone tolerance should not be possible to obtain to any thymus-independent antigen.

SUMMARY

The number of PFC and of RFC was studied in mice which were unimmunized, immunized, or tolerant against lipopolysaccharide of *E. coli* 055:B5 origin. The number of PFC/10⁶ spleen cells increased from 0.5 in normal to 209 in immunized mice. The corresponding figures for RFC were 93 and 513 RFC/10⁶ spleen cells. In tolerant animals, which contained few or no PFC, the number of RFC was increased as compared to that found in unimmunized mice. The formation of rosettes was specific, since their formation was inhibited by soluble coli polysaccharide and by rabbit antisera against mouse immunoglobulins. The antigen-binding cells were not derived from thymus, neither in immune or tolerant mice, because they did not carry the theta antigen. It is suggested that the majority of antigen-binding cells present in tolerant animals are cells having receptors for the antigen of rather low affinity. The relevance of these findings for the induction of high and low zone tolerance is discussed.

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