ANTIGEN RECOGNITION

IV. Discrimination by Antigen-Binding Immunocompetent B Cells between Immunity and Tolerance is Determined by Adherent Cells*

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The antigen-binding cell (ABC)[1] assay has become an increasingly popular index of the presence of immunocompetent bone marrow-derived (B) cells since a nonquantitative correlation was shown between immunocompetence and the antigen-binding phenomenon using ^{125}I-labeled antigen (1, 2). Work by a number of investigators has concentrated on the possible significance with respect to B-cell triggering of surface receptor redistribution by a variety of ligands, notably antireceptor antibodies (3, 4). Such studies have, however, been hampered by the fact that antibody-induced cell differentiation is not readily comparable with antigen-induced immune triggering and that in the latter case a thymus (T)-cell-independent rather than a T-cell-dependent antigen is required to allow for meaningful interpretation of data on antigen-B-cell interaction. Using the T-cell-independent tritium-labeled polymerized flagellar (^3H-POL) H protein of *Salmonella* (labeled by biosynthesis), we have reported earlier that concentrations of this antigen which induce tolerance in vitro bring about inhibition of receptor redistribution on ABC (5). In this communication we shall report on attempts to look for a possible correlation between the induction of tolerance and the dynamics of antigen redistribution across the cell surface membrane. These studies were readily achieved due to the accurate visual localization of the tritiated antigen on the cell surface.

Evidence is presented that the majority of cells binding ^3H-POL undergoes blastogenesis after antigen capping, rapid loss of antigen from the cell, and renewed receptor formation. Under conditions of tolerance induction in vitro (6) with supraimmunogenic concentrations of POL, loss of antigen from the surface membrane was inhibited and the cells remained uniformly labeled for a period of at least 12 h. Such inhibition of antigen redistribution and shedding was reversed, however, by colchicine, in agreement with similar data derived from studies on mitogen-binding cells (7). In spite of this effect on the inhibition of capping, colchicine had no influence on the tolerant state indicating that the binding of tolerogenic amounts of antigen induces unresponsiveness by mecha-

[1] *Abbreviations used in this paper:* A cells, accessory (adherent) cells; ABC, antigen-binding cells; AFC, antibody-forming cells; FCS, fetal calf serum; ^3H-POL, tritium-labeled polymerized flagellin; PBS, phosphate-buffered saline.

nisms other than the mere physical blocking of receptors by antigen. It was indeed possible to support this conclusion by the finding that radioresistant adherent (A) cells (accessory cells) from normal but not from tolerant cell populations could specifically break tolerance. In view of earlier findings from this laboratory on the requirement of A cells in immune responses to T-independent antigens (8), these data ascribe one of the key functions in immunoregulation to A cells.

Materials and Methods

Animals. Male CBA/CaJ mice bred at the Ellerslie Animal Farm, University of Alberta, Edmonton, Canada, 60–90 days of age were used.

Cell Suspensions. Mice were killed by cervical dislocation and their spleens were removed into Leibovitz medium (Grand Island Biological Co., Grand Island, N. Y.). Spleens were cut with fine scissors and the cells were gently pressed through a stainless steel sieve. All suspensions were left standing for 15 min in order to permit clumps of cellular debris to settle out.

Cell Separation. Velocity sedimentation separation was performed in an 18 cm diameter "staput" apparatus, as described previously (9). Cells were applied as a suspension containing no more than 10^7 cells/ml in phosphate-buffered saline (PBS) supplemented with 3% fetal calf serum (FCS). After a sedimentation period of 2.5 h in a buffered step PBS-FCS gradient (5–30%), 15-ml cell fractions were collected and their velocities calculated. The fractions were then appropriately pooled and used for cell transfer or antigen-binding experiments.

Cell Transfer. Mice were given a whole body dose of 950 rads using a gamma-cell 40 cesium-137 source (Atomic Energy of Canada Ltd., Ottawa, Canada) 24 h previous to cell transfer. Cells were resuspended in Leibovitz medium and injected into a lateral tail vein. Injections consisted of 2×10^6 spleen cells in 0.2–0.4 ml of Leibovitz medium. Adoptive immunity was measured on the 7th day after cell transfer. To prevent irradiation death, 10^5 bone marrow cells were injected along with the spleen cells. Adoptive immunity did not occur with the transfer of 2×10^6 bone marrow cells alone.

Preparation of Antigen. Purified polymeric flagellin was prepared from *Salmonella adelaide* flagella (10). ^{3}H-POL was prepared as described elsewhere (5, 11). Sheep erythrocytes (SRBC) were stored in Alsever's solution and washed three times in saline before use.

Immunization. Antigen stimulation consisted of an injection of 20 μg of polymeric flagellin from *S. adelaide* given intravenously to cell donors or intraperitoneally to irradiated cell recipients.

Tissue Culture and Assays of Antibody-Forming Cells. The tissue culture technique has been described in detail previously (11). Briefly, spleen cells were cultured in Eagle's minimal essential medium (MEM) containing 10% vol/vol FCS (GIBCO) in the presence of antigen for 4 days in Marbrook cultures. Antibody-forming cells (AFC) to SRBC were assayed by the method of Cunningham and Szenberg (12) and to POL by the method of Diener (13).

Source of A Cells. A cells were enriched by attachment on plastic Petri dishes as described previously (8). Aliquots of 10^8 cells in 8 ml of Leibovitz medium supplemented with 10% FCS were incubated at 37°C in Falcon plastic tissue culture dishes (100 × 20 mm; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) for 1.5 h. The cells were then resuspended by rocking and incubated for a further hour. Thereafter, the nonadherent cells were suspended and removed. The adherent cells were scraped off with a rubber policeman in the presence of ice-cold Ca^{++}- and Mg^{++}-free PBS containing 0.2% vol/vol sodium EDTA and 5% FCS. The cells were then irradiated with 1,200 rad with a ^{137}Cs source (Gamma cell 40, Atomic Energy of Canada Ltd.) before being used for experiments.

Quantitation of Immunocompetence. The degree of immunocompetence of the cells used to reconstitute irradiated mice is expressed in terms of a reference system. In agreement with other findings (14), a linear log-log dose response curve for serum antibody titers was obtained with respect to the number of normal spleen cells transferred. Hence, serum titers could be related to the cell number of each cell sample transferred in order to calculate the degree of immunocompetence per sample. Serum titers which were obtained from each cell fraction transferred were compared with titers from transferred aliquots of the original unseparated cell suspension (adop-

tive immune response obtained with 10^6 normal spleen cells) in order to assess the percentage of immune reactivity recovered. Results are expressed in terms of the percentage of that fraction which gave the maximum response within a given experiment. Thus all responses were calculated relative to the peak reactivity of each experiment.

Calculations of Velocity Sedimentation Values. Calculations were based on a computer analysis described by Kraft and Shortman (15). The sedimentation velocity was determined by dividing the distance which each cell fraction sedimented through by a time value. This time value comprised the total time the cells in a particular fraction had spent in the cylindrical part of the chamber plus half the time they had spent in the cone section.

Antibody Titers. Antibody titers were performed as described by Armstrong and Kraft (9) using the principle of bacterial immobilization. Assessment was done on a twofold serial dilution assay with 50% immobilization of bacteria as the end point.

Preparation of Rabbit Antimouse Chain-Specific Antibodies. Mouse IgM and IgG were purified from pooled normal mice (CBA/CaJ) serum by ammonium sulphate precipitation, gel filtration on Sephadex G-200, and DEAE-cellulose column. γ-Chain and L chain of IgG were prepared by reduction of disulfide bonds with dithiothreital (16) followed by gel filtration using Sephadex G-200 in 6 M guanidine. μ-Chains of IgM were prepared by the same method as that used for γ-chains after separation of 7S IgM from other macroglobulin. Reduction of 19S IgM to 7S IgM was with cystein followed by gel filtration using Sephadex G-200 in Tris-HCl buffer (0.2 M, pH 8.6) (17). Rabbits were injected with 1 mg of the purified immunoglobulin fragments in complete Freund's adjuvant followed by three further injections intradermally at biweekly intervals in incomplete Freund's adjuvant. Rabbit antimouse chain-specific IgG was purified by ammonium sulphate precipitation, DEAE-cellulose chromatography, and affinity column chromatography using mouse IgM or IgG. No cross reaction was observed by double immunodiffusion between anti-γ and anti-μ after these purifications.

Anti-θ Serum. AKR/J male mice were each immunized with 10 weekly intraperitoneal injections of 10^8 CBA/J thymocytes. The first injection was accompanied by 10^9 killed *Bordetella pertussis* organisms. The mice were bled from the retro-orbital plexus after the 7th wk and finally by heart puncture after the 10th wk. For anti-θ treatment, 20×10^6 cells were incubated for 40 min at 37°C in 1 ml of anti-θ serum of appropriate dilution in Leibovitz medium. After one wash, the cells were incubated again for 40 min in 1 ml of agarose-absorbed guinea pig complement (C', diluted six times in medium). The batch of anti-θ serum used in this work was shown to effectively eliminate T-cell-dependent help in vitro to SRBC. Cytotoxicity for thymocytes was >90% as measured by eosine dye exclusion.

Assay for ABC. The method of detecting antigen bound to cells was essentially the same as that described previously (5, 11). The antigen, ³H-POL, was added to 20×10^6 cells in 1 ml of Leibovitz medium, supplemented with 10% FCS. After 2 h incubation at 4°C, the cells were washed once in Leibovitz medium and twice in a 25–100% gradient of FCS. The cells were then smeared on gelatin-coated slides, fixed in methanol/acetic acid/water (89:1:10) for 30 min, and washed in running water for 10 min. The slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.) and exposed for 21 days at 4°C. Development was in Kodak D-19 developer and giemsa was used for staining. As for immunocompetent cells, the distribution profile of ABC after velocity sedimentation was calculated relative to the peak number of such cells in a specific fraction of each separation experiment.

Results

The Significance of ABC in Immunity: Presence and Significance of ABC in the Spleen, the Specificity of Binding, and Nature of Antigen Receptors. One of the major concerns about the use of ¹²⁵I-labeled antigens is the fact that the frequency of ABC increases over a wide range of increasing antigen concentrations. This was found not to be the case with ³H-POL. Fig. 1 shows the frequency of ABC and the grain count distribution profile after incubation of spleen cells for 1 h at 4°C in the presence of antigen concentrations ranging from immunogenic (200 ng/ml) to tolerogenic (10 μg/ml and more) amounts. The number of ABC per 10^6 spleen cells reached a definite plateau with increasing antigen
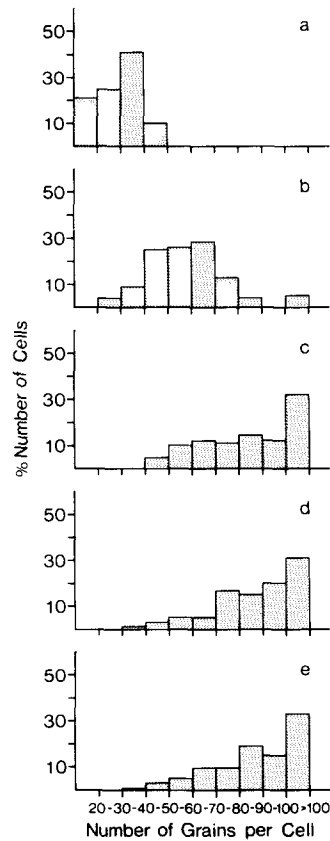
Fig. 1. Grain count distribution profiles of normal mouse spleen cells binding ³H-POL. Cells were incubated in vitro at 4°C for 1 h in the presence of different concentrations of antigen: (a), 200 ng/ml; (b), 1 μg/ml; (c), 5 μg/ml; (d), 10 μg/ml; and (e), 20 μg/ml. The number of ABC in each group per 10⁶ nucleated cells was: 5, 53, 37, 50, and 37, respectively. Significant difference between group (a) and all the other groups. At least 3 × 10⁶ cells were screened for each group.

concentration, suggesting that POL-binding cells comprise a distinct population. There also occurred a shift in the grain count distribution profile from low grain counts at an immunogenic to high grain counts at a tolerogenic concentration.

ABC for ³H-POL were detected in spleen and lymph node at similar frequencies to those described for immunocompetent cells as determined by functional assays (9), i.e., about 20–50 such cells per 10⁶ lymphocytes. To test for the nature of the antigen receptors, spleen cells were incubated with chain-specific antisera at concentrations of 100 μg/ml for anti-μ, 100 μg/ml for anti-γ, and 100 μg/ml for rabbit antimouse anti-L chain. At these concentrations, the subsequent binding of ³H-POL was inhibited to less than 1 cell per 10⁶ lymphocytes by anti-μ or anti-L chain but no inhibition was observed by anti-γ-chain antibody.

It must be noted that upon incubation of cells for a period of 2 instead of 1 h with an antigen concentration of 200 ng/ml the number of ABC was similar to

that obtained with higher amounts of antigen. With lower concentrations of antigen, longer incubation times were required to reach saturation of antigen binding on a per cell basis. The 1 h incubation time used for the study described in Fig. 1 was necessary to avoid excessive labeling of individual ABC at high antigen concentrations for the purpose of grain count analysis. Antigen binding was specific as indicated by the fact that the binding of ^{3}H-POL from *Salmonella* strain 721 was inhibited to <1 cell/10^6 cells by 10 μg/ml of POL from strain 1338 (serological identity for flagellar H antigens) but not from strain 1699 (serologically not identical with strain 1338 for flagellar H antigens).

Immediate Reaction of ABC to Antigen: Cap Formation, Shedding of Antigen, and Production of New Receptors. The key question concerning ABC is their possible identity with immunocompetent cells. The latter should become activated upon antigen binding by undergoing specific functional changes such as blastogenesis and eventually antibody secretion. The fact that POL behaves as an antigen which is independent for its IgM response of T cells (18, 19) makes it an ideal probe to investigate the above question. For this reason, patterns of antigen redistribution on ABC were monitored for their possible biological significance on immune triggering. Incubating antigen-labeled cells at 37°C was accompanied by the now familiar capping phenomenon (Fig. 2 *a* and *b*). In addition, it was observed that prolonged incubation with antigen at 37°C caused ABC to become unlabeled, indicating that antigen had either been pushed off the cell membrane or was released by the cell after pinocytosis (Table I A). The loss of the cap was followed by the appearance of new receptors on the cell surface. This was demonstrated by incubating the cells with labeled antigen after a period of prior incubation with unlabeled antigen. Under these conditions, ABC shortly after they had been stripped of their receptors failed to bind new antigen but would do so within a few hours of culture (Table I B). It therefore appears that ABC were, for the most part, capable of reacting to antigen by forming new receptors.

Late Reaction of ABC to Antigen. Previous work has shown that immunocompetent cells in the mouse can undergo blastogenesis within 24 h of antigenic stimulation (9), whereas only a proportion of ABC which binds ^{125}I-labeled antigen responds in a similar fashion (2). In contrast, studies carried out with tritium-labeled antigen have demonstrated a good correlation between immunocompetence and the presence of ABC. Thus, velocity sedimentation analysis of normal spleen cells revealed a population of ABC (Fig. 3 *a*) and of immunocompetent cells (Fig. 3 *b*) with the characteristics of typical small lymphocytes, indicating that the velocity distribution pattern of the ABC is similar to that of immunocompetent cells detected by adoptive transfer studies. Stimulated spleen cells from mice given antigen 24 h previously contained mainly a population of ABC which was distinguished by larger sedimentation velocities (Fig. 3 *a*) and hence larger cell size (Fig. 2 *c*). Again, immunocompetent cells closely followed the distribution profile of ABC (Fig. 3 *b*). These data are taken to support the notion that ABC for ^{3}H-POL represent B cells which are specifically immunocompetent for this antigen.

The Significance of ABC in Tolerance: Tolerogenic Concentrations of Antigen Prevent Antigen Shedding and the Reformation of Antigen Receptors. A
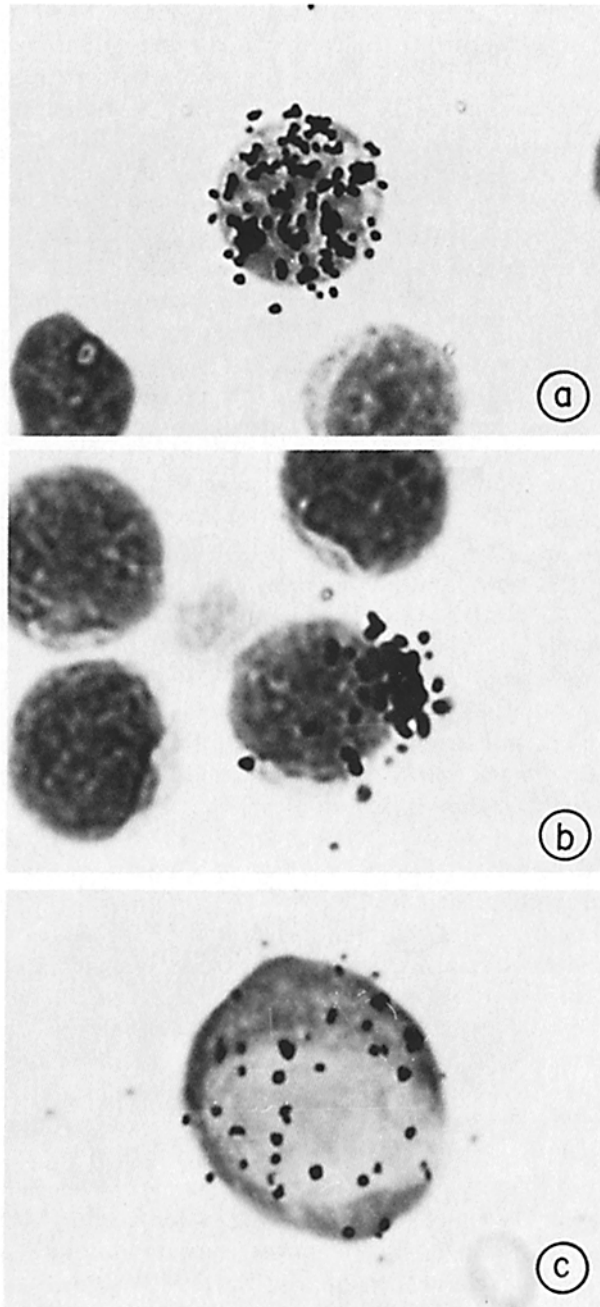
FIG. 2. (a) Small lymphocyte labeled by ³H-POL. (b) Small lymphocyte labeled by ³H-POL which has undergone capping. (c) Large lymphocyte labeled by ³H-POL 2 days after antigenic stimulation. (See also Fig. 3 a and b.) × 2,400.

Table I

Antigen Shedding and the Formation of New Receptors after Antigen Capping

	Time of incubation at 37°C	Labeled cells per 10^6 cells
A*	10 min	24
	1 h	11
	2 h	6
	6 h	3
B‡	2 h	<1
	4 h	8
	6 h	20

* Cells were first incubated at 4°C with ^{3}H-POL for 2 h (500 ng/ml), washed, and further incubated without antigen at 37°C. Samples were taken at 10 min and at 1, 2, and 6 h of incubation and processed for autoradiography. Note: Loss of labeled cells is taken to indicate antigen shedding.

‡ A cell sample was taken from the same pool as in (A), incubated with unlabeled POL at 4°C for 2 h, then at 37°C for 15 min, washed, and then cultured at 37°C. Cell samples were taken at various time intervals of incubation and processed for the binding of ^{3}H-POL. To monitor capping under these conditions, cells from the same source were incubated with ^{3}H-POL for 2 h at 4°C followed by 15 min at 37°C. This resulted in 94% capping of 18 per 10^6 labeled cells. Note: Relabeling of cells is taken to indicate reformation of receptors after antigen-induced receptor stripping.

pertinent question concerns the significance of antigen capping on mechanisms of immune triggering. We have reported elsewhere that antigen-capping inhibition by mitogens occurs independently of immune induction and that capping therefore is not a prerequisite for immune triggering (11). On the other hand, we have observed that concentrations of POL known to induce tolerance in vitro prevent the capping of Ig receptors for this antigen (5). Similarly, others (7) have reported that high concentrations of concanavalin A inhibit the redistribution of surface receptors and that this inhibitory effect could be reversed by agents known to affect microtubular protein, such as colchicine, colcemide, vinblastine, or vincristine. In view of this, we decided to test the possibility that colchicine would also reverse antigen-induced inhibition of receptor capping and, if so, whether such an effect would coincide with reversibility of the tolerant state.

Normal mouse spleens were incubated with either a tolerogenic concentration of 40 μg/ml[2] or an immunogenic concentration of 500 ng/ml of POL at 4°C in the presence or absence of colchicine at a concentration of 10^{-4}M, and the fate of the surface-bound antigen followed by autoradiography. In order to prevent poor quality of autoradiographic preparations due to excessive radioactive labeling of ABC at high antigen concentrations, lymphocytes were first prelabeled by incubation with 500 ng/ml of ^{3}H-POL for 2 h at 4°C, followed by another 2 h

[2] Although less antigen (i.e., 10-40 μg/ml) is sufficient to induce tolerance, the most efficient inhibition of capping of ABC occurred at an antigen concentration of 40 μg/ml.
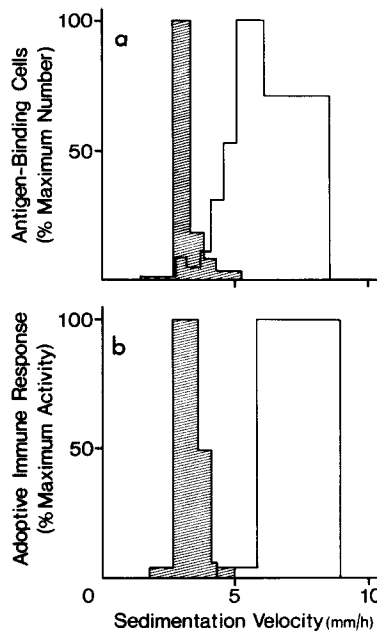
Fig. 3. (a) Distribution profile of ABC before and 2 days after antigenic stimulation. Spleen cells from two mice were pooled and 405 × 10^8 cells were sedimented. Hatched area, unstimulated spleen cells, and open area, spleen cells from animals injected with antigen 2 days previously. Note: Nearly the entire population of ABC is found among the blast cells in stimulated, but among small lymphocytes in unstimulated animals. (b) Distribution profile of immunocompetent cells before and 2 days after antigenic stimulation. Spleen cells from two mice were pooled and 405 × 10^8 cells were sedimented. Immunocompetence was assessed by the transfer of 2 × 10^6 spleen cells to irradiated recipients (see Materials and Methods). Hatched area, unstimulated spleen cells; and open area, spleen cells from animals injected with antigen 2 days previously. Note: Nearly the entire population of immunocompetent cells as well as of ABC is found among the blast cells in stimulated, but among small lymphocytes in unstimulated animals.

incubation at 4°C in the presence of either 500 ng/ml or 40 μg/ml of unlabeled POL. Using this procedure grain counts on autoradiographies of labeled cells were comparable between the test group and the control. As shown in Table II, capping and shedding of antigen by ABC was inhibited under tolerogenic conditions, i.e., in the presence of 40 μg/ml of POL. Colchicine reversed this inhibition. In comparison to the experiments recorded in Table I it is noteworthy that in this experiment the process of antigen shedding was almost complete after incubation of the cells at 37°C for as short a period as 30 min. We have reported earlier in this paper that, after the loss of antigen from the cell, new receptors appear on its surface. Interestingly, such receptor reformation also took place under conditions at which inhibition of capping by tolerogenic amounts of POL bound to the cell was reversed by colchicine. This was shown by an experiment on antigen redistribution analogous in timing to our routine procedure for tolerance induction in vitro (6). ABC were exposed to either an immunogenic or tolerogenic concentration of nonradioactive POL for 6 h at 37°C in the presence of colchicine at a 10^{-4} M concentration. The cells were then

TABLE II

Effect of Colchicine on Antigen Redistribution

	Antigen ^{3}H-POL	Labeled cells/10^6 (4°C)	Caps after 15 min (37°C)	Labeled cells/10^6 after 30 min at 37°C
			%	
Control	500 ng/ml (immuno-genic)	37.1	92	7.0
	40 μg/ml (tolerogenic)	43.2	15	32.0
With colchicine, 10^{-4} M	500 ng/ml	34.1	81	6.7
	40 μg/ml	34.8	>99	<1

Normal spleen cells were incubated with different concentrations of radiolabeled POL for 2 h at 4°C in the presence or absence of colchicine. After washing, colchicine was readded and the cells exposed to 37°C. A sample was taken after 15 min to assess the degree of capping and after 30 min to test for the number of labeled cells. Experiment repeated three times with similar results. Note: Capping and shedding of antigen is inhibited under tolerogenic conditions. Colchicine reverses this inhibition.

washed and cultured for 12 h without antigen. After such time, they were re-exposed to ^{3}H-POL for 2 h at 4°C in order to label newly formed receptors analogous to the procedures used for the experiment reported in Table I. Surprisingly, colchicine not only caused reversibility of capping inhibition by tolerogenic amounts of POL bound, but it enabled the cells to reform new receptors, as was the case for controls (Table III). It was therefore of interest to ascertain whether these cells had also regained the capability to participate in a normal immune reaction upon antigen challenge in vitro. Normal spleen cells were therefore incubated for 6 h with a tolerogenic (40 μg/ml) or immunogenic (100 ng/ml) concentration of POL in the presence or absence of colchicine. After this preincubation period, the cells were washed four times and cultured for 4 days with an immunogenic amount of POL (100 ng/ml). For specificity control, sheep erythrocytes at a concentration of 0.01% were added along with POL. Results from assessing the number of AFC at the end of the culture period are shown in Table IV A. In spite of colchicine causing antigen shedding and receptor reformation, it failed to affect the state of tolerance.

Radioresistant A Cells Abrogate Tolerance In Vitro. We have reported elsewhere that high doses of corticosteroids cause a marked shift in the triggering threshold with respect to the antigen concentration necessary to induce tolerance in vitro (20, 21). Since this effect could be abrogated by radioresistant A cells from either an adherent population of spleen cells or from peritoneal exudate cells, we wondered whether A cells would also influence the state of in vitro tolerance when using normal cells.

Experiments were carried out in which normal mouse spleen cells were incubated in vitro for 6 h in the presence of either an immunogenic (100 ng/ml) or a tolerogenic (40 μg/ml) concentration of POL with or without colchicine in accordance with the experiment reported in Table IV A. SRBC were added as specificity control together with the flagellar antigen. After washing four times,

TABLE III
Effect of Colchicine on Receptor Reformation

	Preincubation: 2 h at 4°C, 30 min at 37°C	
	Antigen (POL)	ABC/10⁶ cells
Control	250 ng/ml (immunogenic)	33
	40 μg/ml (tolerogenic)	<1
Colchicine, 10⁻⁴ M	250 ng/ml	40
	40 μg/ml	30

Normal spleen cells were incubated with either immunogenic or tolerogenic concentrations of POL for 2 h at 4°C, in the presence or absence of colchicine. After washing, colchicine was readded and the cells exposed to 37°C for 30 min. After a second wash they were cultured in the absence of antigen and of colchicine for 12 h. Thereafter the cells were incubated with ³H-POL for assessment of ABC. Experiment repeated three times with similar results. Note: Reformation of receptors is inhibited under tolerogenic conditions. Colchicine reverses this inhibition.

5 × 10⁶ normal A cells irradiated with 1,200 rads and treated with antitheta serum and complement were added and the mixture cultured for 12 h, after which time an immunogenic concentration of POL was added. In another experiment, adherent spleen cells which had been irradiated with 1,200 rads were preincubated for 6 h with either 30 μg/ml or 100 ng/ml of POL, together with SRBC. After this incubation period, they were washed four times. Again, 15 × 10⁶ tolerant or immune spleen cells were then admixed with 5 × 10⁶ A cells which had been preincubated with an immunogenic or tolerogenic concentration of POL. The cells were then cultured for 4 days in the presence of an immunogenic dose of the antigen. Much to our surprise it was found that A cells were indeed capable of breaking tolerance regardless of whether or not colchicine was present during its induction (Table IV B). Furthermore, A cells which had been preincubated with immunogenic amounts of POL were capable of breaking tolerance, whereas A cells preincubated with tolerogenic concentrations of the antigen failed to do so (Table V). It must be emphasized that "tolerant" A cells, although they failed to reverse unresponsiveness to POL were equally capable of cooperating in a response to SRBC, as were "immune" A cells. Irradiated A cells, when cultured alone, were incapable of generating an immune response.

Discussion

The use of intrinsically tritium-labeled antigen is an important refinement of the ABC assay as it permits the elimination of artifacts and allows one to follow, with a high degree of accuracy, the redistribution dynamics of cell surface-bound antigen (5, 11). Our data indicate that lymphocytes in the normal mouse spleen capable of specifically binding POL, possibly by IgM receptors, may indeed represent a distinct population of cells. We surmise this from the data which indicate a plateau effect concerning cell numbers when lymphocytes were incubated with increasing concentrations of the antigen.

TABLE IV
Effect of Colchicine and Adherent Cells on Tolerance In Vitro

Spleen cells (15 × 10⁶/ml) preincubated for 6 h with:		A cells anti-θ + C′* (1,200 rads)	AFC/culture ± SEM	
Antigen POL + SRBC	Colchi- cine		POL	SRC
	M			
A‡ 100 ng/ml	–	–	2,040± 100	3,000± 58
40 μg/ml	–	–	70± 20	3,193± 580
100 ng/ml	10⁻⁴	–	2,650± 495	3,306± 470
40 μg/ml	10⁻⁴	–	100± 14	4,193± 425
B§ 100 ng/ml	10⁻⁴	5 × 10⁶	2,631± 174	5,525± 746
40 μg/ml	10⁻⁴	5 × 10⁶	1,320± 83	8,687± 681
100 ng/ml	–	5 × 10⁶	4,313± 575	5,392± 275
40 μg/ml	–	5 × 10⁶	4,080± 567	5,700± 591
100 ng/ml	–	20 × 10⁶	10± 3	12 ± 4

* Treatment with antitheta serum plus complement. Such treatment of normal spleen cells was shown to abolish a subsequent response to SRBC (see Materials and Methods). Experiment repeated three times with similar results.

‡ Normal spleen cells were preincubated for 6 h at 37°C with a tolerogenic or immunogenic concentration of POL and 0.01% SRBC in presence or absence of colchicine. After washing they were cultured for 12 h and then challenged with an immunogenic concentration of POL. The immune response was measured 4 days later. Note: Colchicine failed to reverse tolerance to POL.

§ Normal spleen cells were treated as in (A) except that irradiated and anti-θ + C′-treated A cells were added after the 6 h preincubation period. Note: Tolerance to POL was reversed by A cells regardless of treatment with colchicine.

The biological significance of the antigen-binding event is indicated by the subsequent changes observed in the labeled cell population. Thus the majority of ABC was quick to form a "cap" of antigen which was then shed within a period as short as 30 min (Table II) to 4 h (Table I). Although we have no conclusive evidence that POL ever becomes pynocytosed, as reported by others when using different ligands for surface receptors (4, 22), we regard such an event as unlikely to occur because of the sometimes very rapid disappearance of label from the cell. Even if the antigen had undergone degradation intracellularly within such a short period, it is likely that broken down products (tritiated leucin) of the biosynthetically labeled antigen would have still been found labeled in the cell and would possibly have been reutilized. After antigen shedding, the cells were able to form new receptors within a period of 4–6 h. This is at variance with reports by others (23) who have shown the reappearance of receptors following capping after a period of 18 h. This difference in timing may result from the fact that in their studies T-dependent antigens were used, whereas POL has been shown to be T-independent in vitro (18, 19). The finding that the majority of ABC can react to antigen by cap formation, cap shedding,

TABLE V

The Capability of Accessory (A) Cells to Reverse Unresponsiveness to POL In Vitro

Spleen cells (15 × 10⁶) preincubated for 6 h with POL + SRC	A cells added (1,200 rad)*		AFC/culture ± SEM	
	"Tolerant"	"Immune"	POL	SRC
100 ng/ml	—	—	6,327 ± 227	7,383 ± 240
30 μg/ml	—	—	487 ± 30	4,217 ± 562
100 ng/ml	—	5 × 10⁶	5,460 ± 227	9,233 ± 1,500
30 μg/ml	—	5 × 10⁶	5,507 ± 447	9,677 ± 281
100 ng/ml	5 × 10⁶	—	3,347 ± 35	8,342 ± 586
30 μg/ml	5 × 10⁶	—	493 ± 45	4,717 ± 374
100 ng/ml		20 × 10⁶	14 ± 14	1.3 ± 1

Normal spleen cells were preincubated for 6 h at 37°C with a tolerogenic or an immunogenic concentration of POL and 0.01% SRBC. After washing, washed preincubated A cells were added and the cell mixture challenged with an immunogenic concentration of POL + 0.01% SRBC for a period of 4 days. At such time cultures were tested for immune responses to the two antigens. Experiment repeated four times with similar results.

* Tolerant A cells, after preincubation for 6 h with 30 μg/ml of POL + SRBC (0.01%); immune A cells, after precinbuation for 6 h with 100 ng/ml of POL + SRBC (0.01%). Note: Immune but not tolerant A cells are capable of breaking unresponsiveness.

and then by the production of new specific receptors indicates that passively acquired antibody probably does not interfere with the assay. In addition, we have provided supportive evidence which indicates that such cells undergo blastogenesis after antigenic stimulation. This is deducted from the close correlation that exists between the presence of ABC in a lymphocyte population and that of immunocompetent cells before and after antigenic stimulation, as revealed by velocity sedimentation techniques. In contrast to our earlier studies using ^{125}I-labeled POL (2), this suggests that the majority of ³H-POL binding cells may indeed represent immunocompetent cells. The only explanation at hand for this difference must refer to the different labeling techniques used and the different sensitivities in the detection of the two isotopes. ^{125}I-labeling involves oxidation of the antigen, a procedure which may alter the configuration of the protein and hence change its binding characteristics. The use of a biosynthetically labeled antigen excludes such a possibility.

We have reported earlier (5) that ABC under conditions known to induce tolerance in vitro (6) failed to cap the antigen. A more formal test of this observation has reconfirmed this point and has furthermore provided evidence that such cells fail to form new receptors, even if cultured for a prolonged period of time in the absence of antigen. Possibly, their receptors remained blocked by the antigen, as suggested by others in a different experimental system (24). There has been considerable interest concerning the question as to whether the tolerant state of a B cell is maintained by the continuous presence of antigen on its surface (receptor blockade) (24) or whether its initial encounter with the cell mediates a tolerogenic signal to the protein synthetic machinery. Our experiments on the reversibility induced by colchicine of the antigen capping and

shedding inhibition under tolerogenic conditions may help in answering this question. The data indicate that tolerant cells do carry antigen on their surface for a prolonged period of time, and that, however, antigen shedding can be brought about by treatment of the cells with colchicine; this is in analogy with similar findings by other investigators (7) on the immobilization of cell surface receptors when using high concentrations of concanavalin A as a ligand. Most important, however, the cells which had reversed antigen-induced inhibition of receptor mobility by colchicine proceeded to reform new receptors but were still unable to respond to an otherwise immunogenic challenge by POL. That this unresponsiveness was not due to the presence of colchicine during tolerance induction is indicated by the fact that the control group which was treated with immunogenic amounts of POL together with colchicine subsequently gave a normal immune response.

The data thus suggest that the induction of tolerance under the conditions described is not due to "receptor blockade" but represents an antigen-induced process which renders the cell unresponsive either directly via its own metabolic pathways or through another cell class. Our reconstitution experiments reported here favor the second possibility without excluding the first one. Thus, the state of unresponsiveness in spleen cells after preincubation with tolerogenic amounts of POL could be reversed by radioresistant A cells which had or had not been preincubated with immunogenic concentrations of POL. A cells, however, after incubation with a tolerogenic amount of the antigen, were incapable of breaking unresponsiveness to POL, although they cooperated normally in the generation of an immune response to SRBC. It is possible that preparations of A cells from the spleens of normal mice could have been contaminated with immunocompetent cells. Such precursor cells are known to be highly radiosensitive, however, and would therefore have been functionally eliminated by irradiation. Furthermore, the possibility of having triggered the generation of suppressor T cells during the induction of tolerance has been excluded by the fact that treatment of A cells with anti-theta serum and complement was without effect. Furthermore, tolerance as well as immunity to POL may be induced in vitro in the absence of T cells (unpublished data).

It has been suggested that one of the functions of A cells might be the removal of antigen from the environment of immunocompetent cells in order to minimize its tolerogenicity due to high concentrations (25). This theory is contradicted by our finding that colchicine brought about the capping and shedding of tolerogenic amounts of antigen from B cells without affecting the state of unresponsiveness (Table IV A). Furthermore, A cells reversed tolerance independently of the colchicine-induced removal of surface-bound antigen (Table IV B).

Most investigators believe that tolerance results from the direct interaction of antigen with immunocompetent cells, implying that the antigen exerts a tolerogenic signal as distinct from an immunogenic signal via the surface membrane to the cell's machinery. Although our data do not directly disprove this hypothesis, they raise the possibility that tolerant B cells may not even exist. It is at least intriguing that B cells, after antigen-induced inhibition of capping had been reversed by colchicine, still remained refractory to immune triggering unless confronted with A cells from a normal spleen cell population. The

possibility has to be taken into account therefore that under the experimental conditions used, unresponsiveness was due to the lack of effective cooperation between A cells and immunocompetent B cells rather than to a functional deficiency at the level of the B cells per se. This interpretation is further reinforced by the finding that the reversibility of capping inhibition in tolerant cultures is not a prerequisite for the breaking of the unresponsive state (Table IV B).

We have shown earlier that in vitro induced tolerance to POL may be reversed within a critical period of 2 days after induction by treating tolerant cells with trypsin (26) with cortisone and mercaptoethanol (21), or by transferring them to lethally irradiated recipients (26). Current work in our laboratory suggests the possibility that the first two types of treatment act via A cells.[3] Likewise, in view of our data reported here, the breaking of tolerance upon in vivo transfer of cells (26) is most probably due to the activity of radioresistant A cells of the recipient.

Besides the described phenomenon of A-cell "tolerance" the possibility exists therefore that tolerogenic amounts of antigen adversely affect the physiological viability of the immunocompetent B cell by blocking its surface due to inhibition of antigen capping and shedding. Hence our finding that tolerance to POL in such cells remains reversible for a period of 2 days only (26).

The data most difficult to explain concern the apparent specificity of A-cell-B-cell cooperation. Specificity is inferred from the fact that tolerance to POL is antigen specific and that it can be broken by "immune" but not by "tolerant" A cells. This conclusion is also supported by recent experiments in which normal A cells were capable of restoring immunocompetence in A-cell-deficient spleen cell cultures to both SRBC and POL, whereas restoration by A cells from spleens tolerant to POL occurred only with respect to SRBC but not to POL. Furthermore, we have shown that the antigen specificity of "tolerant" A cells is not only valid relative to T-cell-dependent SRBC antigens but also to T-cell-independent dinitrophenylated Ficoll.[3] In our view the most acceptable explanation of the phenomenon concerns the possibility that POL becomes immunogenic only when processed by A cells. Such processing may involve the association of the antigen or parts of it with cellular products (superantigens). That such a product may even be coded for by Ia genes is inferred from recent work by other investigators (27) on cooperation mechanisms between T cells and A cells. It is conceivable that too high a concentration of antigen would adversely affect the configuration of such an antigen-cell factor complex and thus render it nonimmunogenic or tolerogenic for B cells. That A cells may present POL to B cells via the cell surface has been suggested for T-dependent antigens. Such a possibility is unlikely to apply to POL in view of our failure to demonstrate surface-bound POL on adherent macrophage-like cells (assuming that the latter do indeed satisfy the functional definition of A cells).

Although some of the phenomena described in this communication are difficult to explain at the basic level without involving a great deal of ad hoc speculation, they are likely to be of importance in the search for an understand-

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[3] Lovchik, J., K.-C. Lee, and E. Diener. Manuscript in preparation.

ing of immune triggering mechanisms. The hitherto held belief that so-called T-independent antigens are capable of triggering B cells directly has led to competing theories concerning immune induction (28). Our evidence for a decisive role of A cells in the triggering of B cells by both T-dependent and T-independent antigens (8) and, as shown in this communication, on the induction of B-cell tolerance by T-independent antigens, calls for a critical reassessment of current concepts of immune induction.

Summary

Mouse spleen cells capable of specifically binding intrinsically tritium-labeled polymerized flagellin (POL) (labeling by biosynthesis of flagellar protein) via IgM receptors were found to comprise a distinct population of about 20–50 cells per 10^6 lymphocytes. Evidence is presented that the majority of mouse spleen cells binding tritium-labeled POL undergoes blastogenesis after antigen capping, antigen shedding, and receptor reformation. Under conditions of tolerance induction in vitro, however, loss of antigen from the cell surface was inhibited. Such inhibition of antigen redistribution and shedding was reversed by a short pulse of colchicine and new antigen receptors were formed. In spite of this, colchicine had no effect on the tolerant state. However, tolerance could be broken, regardless of presence or absence of the alkaloid, with radioresistant theta-negative accessory (A) cells (adherent cells) from normal but not from tolerant spleen cell populations. "Tolerant" A cells, although they were incapable of cooperating in a response to POL, were capable of participating in a response to a second unrelated antigen. It is concluded that tolerance to POL in vitro is induced by mechanisms other than the physical blocking of bone marrow-derived (B) cell receptors by antigen. Most likely, the discrimination by the B cell between a tolerogenic and immunogenic signal is mediated by A cells.

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