A NATURAL MODEL OF IMMUNOLOGIC TOLERANCE

Tolerance to Murine C5 Is Mediated by T Cells, and Antigen Is Required to Maintain Unresponsiveness*

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The mechanism whereby the immune system is rendered unresponsive to self is one of the central problems of immunology. When natural tolerance, which is more physiological and long lasting than any other form of experimentally induced unresponsiveness fails, autoimmune disease may develop. But the mechanism of natural tolerance has been difficult to study, not only because suitable models are lacking, but also because the presence of the antigen in the host has hampered the detection of autoantibodies. In the few models of natural tolerance to soluble protein antigens examined, for example thyroglobulin or alpha-fetoprotein, it has been suggested that T cells are unresponsive, whereas B cells are not (1–3). However, these antigens circulate in the blood in minute amounts, and the lack of B cell tolerance might simply reflect a difference in dose requirements for induction of T-vs.-B cell tolerance (4). Furthermore, autoimmunity to thyroglobulin, as studied in a T cell proliferation assay (5), challenges the state of T cell tolerance to this T-dependent antigen. On the other hand, B cells are known not only to bind autoantigens (6), but also to produce autoantibodies either after immunization with cross-reactive self antigens or stimulation with a mitogen such as lipopolysaccharide (2, 3, 7, 8). Whether natural B cell tolerance had been broken or simply did not occur is unclear. In contrast, it is known that both T and B cells can readily be rendered unresponsive in experimentally induced unresponsiveness to either soluble heterologous antigens or to haptenated isologous gamma globulin conjugates (4, 9–11).

Ideally, one should be able to assess the state of tolerance of immunocompetent cells in their environment either with or without the presence of the antigen under conditions where autoimmunity does not normally occur. To fulfill this goal, we selected the MUB1 antigen, which is the murine fifth component of complement C5 (12). Murine C5, like its human counterpart (13), consists of two polypeptide chains (14) and has a serum concentration of 50–85 µg/ml (12), which is more than 100-fold greater than that of thyroglobulin or alpha-fetoprotein.

Two strains of mice, B10.D2OSN (C5 deficient) and B10.D2NSN (C5 sufficient), are congenic, differing only at the MUB1 locus. This allows studies of tolerance to C5 after reciprocal transplantation of immunocompetent cells into irradiated hosts.

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without the development of either graft-vs.-host or host-vs.-graft reactions. We used two complementary approaches. In the first, lymphoid cells from either strain were transferred into irradiated C5-deficient hosts, where detection of anti-C5 antibody upon challenge with murine C5 was possible because of the absence of the antigen. Second, lymphoid cells from either strain were transferred to irradiated C5-sufficient hosts, whose native C5 provided the antigenic stimulus. The results showed that T but not B cells were unresponsive and that the C5 antigen was required to maintain unresponsiveness.

Materials and Methods

**Animals.** Male 6–8-wk-old B10.D2OSN/J, B10.D2NSN/J A/J, A/HeJ, C57Bl/6J by DBA/2J (BDF1) mice were obtained from The Jackson Laboratory, Bar Harbor, ME and maintained at the Children's Hospital animal facilities. On arrival, all B10.D2 mice received water acidified with HCl to pH 3.0 for 5 d, then tetracycline (Rachelle Laboratories, Long Beach, CA) water for 3 d. Mice that were intended as irradiated hosts were medicated for an additional 5 d with acid water plus 20 μg tobramycin/ml before irradiation.

**Antigens.** A mouse C5 preparation was obtained from the serum of B10.D2NSN mice. Male B10.D2NSN mice older than 12 wk were used because serum C5 levels rise with age in male mice (12). The serum donors were tail bled, and their blood was pooled by pairs and allowed to clot at room temperature for ~3 h. After the 3-h clotting time, the serum was centrifuged at room temperature and stored at −20°C. The acid euglobulin fraction of this serum, containing C5, was prepared as described by Cinader et al. (15). The euglobulin was resuspended in 0.15 M phosphate-buffered saline (PBS), pH 7.0, in a volume 1/10th that of the original serum. The solubilized euglobulin was combined with complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI), and the mice were injected intraperitoneally in a 0.2-ml volume. Assuming a minimum C5 concentration of 50 μg/ml of serum, each mouse received ~50 μg.

Ovalbumin was purchased from Sigma Chemical Co., St. Louis, MO. Immunizations with ovalbumin were with 50 μg injected intraperitoneally in CFA in a final volume of 0.2 ml.

**Radiation Transfer Protocols.** B10.D2 mice were given a dose of 760–780 rad in a split dose from a Cesium Gammacell (General Electric Co., Instruments Products Section, West Lynn, MA). Doses were separated from 3–5 h to allow recovery of the gut epithelium but not of the bone marrow. This dose was lethal without reconstitution for B10.D2OSN but not for B10.D2NSN mice, presumably because of differences in susceptibility to infection in these mice.

**Cell Preparations for Transfers.** Whole spleen cell suspensions were prepared by washing spleen cells three times in minimal essential medium (MEM) at 4°C. 70 × 10⁶ cells were injected intravenously per mouse.

Nonadherent spleen cells were prepared by adherence of whole spleen to Falcon plastic petri dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) at a concentration of 20 × 10⁶ cells/ml for 90 min at 37°C in MEM with 2 mM glutamine and 10% fetal bovine serum (FBS). The nonadherent cells were removed by gently swirling the plates. These cells were further washed three times in MEM as for whole spleen. 70 × 10⁶ cells were injected per mouse.

Anti-Thy-1.2 plus complement-treated cells were prepared as follows. The viable cells were counted and spun into a pellet. They were resuspended in 1 ml of monoclonal anti-Thy-1.2 (the kind gift of Dr. Ann Marshak-Rothstein) diluted to 1/100 per 250 × 10⁶ viable cells. The cells were incubated for 30 min on ice, then washed twice in MEM and resuspended in 10 ml of guinea pig complement (GPC), 1 part GPC, 3.5 parts MEM plus 10% FBS, and 0.5 parts distilled H₂O per 250 × 10⁶ cells by the original count. The cells were incubated for 45 min at 37°C. The cells were then washed three times in MEM before injection.

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1 Abbreviations used in this paper: C5, murine fifth component of complement; CFA, complete Freund's adjuvant; EACA, epsilon amino caproic acid; FBS, fetal bovine serum; GPC, guinea pig complement; GVB, gelatin veronal buffer; MEM, minimum essential medium; PBS, phosphate-buffered saline; PBS-BSA, phosphate-buffered saline in bovine serum albumin; RBC, erythrocytes; SRRBC, sensitized rabbit erythrocytes.
Cell Fractionation. Surface Ig+ cells were prepared after Parish (16) as follows. As a separating reagent, affinity-purified rabbit anti-mouse immunoglobulin F(\(ab')_2\) fragment was prepared. The starting material was a rabbit antiserum to the RPC5 tumor product (gamma 2a K). This antiserum was shown by immunoelectrophoresis to have specificity for other mouse immunoglobulins, including IgM and IgG1. The antiserum was affinity purified on Sepharose 2B (Pharmacia Fine Chemicals Inc., Div. of Pharmacia Inc., Piscataway, NJ) coupled to a 50% ammonium sulfate fraction of B10D2OSN serum using the cyanogen bromide technique (17). The adsorbed antibody was eluted from the column with 0.2 M glycine, pH 2.2. The F(\(ab')_2\) fragment of the affinity-purified antibody was generated by digestion with pepsin (Worthington Biochemical Corp., Freehold, NJ) in 0.1 M Na acetate buffer, pH 4.55, 20 mg pepsin/g protein. The digestion was for 36 h at 37°C. The reaction was stopped by bringing the pH to 8.0 with NaOH. The digest was applied to a Sephadex G150 column (dimensions, 75 × 1.75 cm) run in normal saline. No undigested protein was observed, but a symmetrical peak of F(\(ab')_2\) was observed, and a sizeable peak of partially digested Fc fragments followed. The F(\(ab')_2\) fragment containing peak was pooled and used. The F(\(ab')_2\) reagent was coupled to sheep erythrocytes by the CrCl~ technique (17). The coupled cells were used the day after coupling, after extensive washing. Spleen cells to be fractionated by rosetting were washed three times in cold MEM and incubated at 37°C for 90 min in MEM with 2 mM glutamine and 10% FBS. This incubation was done to allow cells to shed passively acquired immunoglobulin. The pre-incubated cells were washed twice in MEM and counted. Rosetting was performed by the method of Parker (18). The B cell preparation from the rosetted pellet was only 6% Thy-1.2⁺; the T cell preparation from the interface was 81% Thy-1.2⁺.

Assay of Mouse C5 and Anti-Mouse C5. To rapidly, conveniently, and accurately measure mouse C5 levels with a high sensitivity, we developed an assay for mouse C5 that uses sensitized rabbit erythrocytes as targets, C5-deficient mouse serum as a source of all other complement components, and dilutions of normal mouse serum as source of C5. The assay can measure C5 in dilutions of mouse serum up to 1/200,000, and in human and guinea pig serum at lower sensitivity.

Rabbit erythrocytes were used because mouse spleen serum has been reported to be very inefficient in lysis of sensitized sheep erythrocytes (19). We confirmed that observation. We therefore hypothesized that rabbit erythrocytes, which are also permissive for alternative pathway lysis (20), might be a better target. In fact, C5-sufficient mouse strains do lyse unsensitized rabbit erythrocytes rather well. However, lysis of unsensitized rabbit erythrocytes by fresh A/HeJ serum reconstituted with small amounts of mouse C5 was variable and depended on the batch of A/HeJ used. This variability could be removed by sensitizing the rabbit RBC with a 4-d A/HeJ anti-rabbit RBC serum. Thus, the variability in lysis was probably the result of a lack of natural antibody in some batches of A/HeJ.

The C5 Assay. Rabbit RBC stored at 4°C in Alsevier's solution (0.114 M glucose, 3.2 mM Na citrate, 0.012 M NaCl), 0.012 M NaCl) were washed four times in gelatin veronal buffer (0.1% gelatin, 5 mM sodium barbital, 2 mM MgCl₂, pH 7.3; GVB) and sensitized with A/HeJ anti-rabbit RBC. This antibody was prepared by immunizing old A/HeJ males with 0.2 ml of 10% rabbit RBC in saline intraperitoneally and bleeding on day 4. The antiserum was titered to determine the appropriate concentration. Often very high concentrations were completely inhibitory. The optimum sensitizing concentration was always just below the end-point in hemagglutination, usually 1/400 and 1/1,000. For the assay, 1 ml of 1% rabbit RBC was added to 5 ml of antibody in GVB at room temperature, mixed, and incubated at room temperature for 15 min. The sensitized rabbit RBC (sRBC) were washed twice in GVB and resuspended to 0.5% in GVB for use. The cells were passed through a 21-gauge needle before use, to disrupt any small aggregates that would detract from the accuracy of the assay. A/HeJ males were bled within 2 h of assay time. The blood was pooled on ice to the desired concentration and allowed to clot at 4°C for 30 min. The clot was then spun out at 2,000 rpm for 15 min at 4°C. The serum was diluted 1/4 for use in GVB. Serum to be assayed for C5 was obtained within 3 h of assay time. The serum was allowed to clot for up to 30 min at room temperature, then until needed at 4°C. In the assay, 100 μl of serum dilution to be tested, 100 μl of C5-deficient serum (at 1/4), and 60 μl of sRBC (at 0.5%) were combined on ice. The sRBC were always added last. The assay was performed in new 10 × 75-mm borosilicate tubes (CMS Div., Instrumatics, Inc., Houston,
The assay tubes were mixed when complete and incubated at 37°C in a shaker bath for 1 h. After the incubation, the assay tubes were mixed, spun at 2,000 rpm for 7 min at 4°C, and the supernatants removed to dilution tubes as expeditiously as possible. Duplicate supernatants were pooled together, using 175 µl from each tube and 525 µl of saline. The dilution tubes were stored overnight at 4°C. The optical density at 412 nm was read the following day. Results were computed as Z (sites/cell) by the formula: Z = -ln(1 - lysis/total lysis). An optimized version of this assay, using EAC14 as target cells, has been described by Ooi et al. (21).

The Anti-C5 Assay. Inhibition of C5 lytic activity was used as an anti-C5 assay. The assay was performed analogously to the C5 assay, except that the C5-deficient serum was reconstituted with C5 to a starting concentration of 1/25,000 of C5-sufficient mouse serum, and the 100 µl of C5 dilution was replaced with 100 µl of antiserum dilution. The antisera had to be heat inactivated (in their dilution of highest concentration at 54°C for 12-15 min to remove active complement components. The sera could not be assayed at a concentration higher than 1/25 because of anticomplementary activity. The anticomplementary activity made it necessary to run many normal serum controls, as activity in such sera could range from 0-40% inhibition at 1/25.

Immunodiffusion. Anti-C5 was also assayed, with much less sensitivity than the above method, by immunodiffusion. The 1% agar (Difco Laboratories, Detroit MI) was made 0.1 M in epsilon amino caproic acid (EACA) and 10 mM in EDTA (pH adjusted to 7.0). The buffer was 0.025 M barbital buffer, pH 8.4. To see good precipitin lines, both C5-sufficient serum and antiserum had to be applied to the wells twice. Immunoelctrophoresis kits (Gelman Sciences, Inc., Ann Arbor, MI) were used for the immunodiffusions. In general, any antiserum that produced a line in immunodiffusion had >80% inhibition of C5 hemolytic activity at 1/25. A very strong antiserum caused detectable inhibition at 1/2,500.

Hemagglutination. Anti-ovalbumin was assayed by hemagglutination. SRBC were tanned with ovalbumin using the CrCl3 technique. Ovalbumin was used at 0.5 mg/ml. 50 µl of 2% tanned SRBC was added to 50 µl of antibody solution and the direct titers after mixing and settling. The patterns were then washed in 100 µl of diluent and resuspended in 100 µl of 0.5% rabbit anti-mouse immunoglobulin. The patterns were allowed to reform, and the indirect titer was recorded. Diluent was phosphate-buffered saline, 0.03 M phosphate, 0.15 M NaCl, pH 7.0, 2.5 mg/ml in bovine serum albumin (PBS-BSA).

Statistical Analysis. For C5 inhibition levels and C5 levels, arithmetic means and standard deviations were calculated. Individual positive responses were determined as those values above the mean plus two standard deviations of the control group. Groups were compared by Student’s t test.

Results

C5 Assay. Fig. 1 shows the titrations of B10.D2NSN serum (homozygous C5 sufficient), BDF1 serum (heterozygous C5 sufficient), guinea pig serum, and human serum. The assay was most efficient in the detection of mouse C5 activity. BDF1 mice had approximately one-half the activity of the homozygous strain. The slope of the plot of log Z vs. log C was never >1.1, but, with mouse serum as the source of C5, the slope varied from titration to titration, from 0.7 to 1.0. The reason for this variation is unknown. The fact that the slope did not generally exceed 1.0 indicates that no more than one complement component was titrated. C5 was easily detected at a dilution of 1/200,000 of normal mouse serum. Assuming a concentration of 50 ng/ml in serum, this result represents a concentration of 0.25 ng/ml which is the minimum detectable by that assay. Serum could not be reliably assayed at concentrations above 1/10 because of anticomplementary activity.

Anti-C5 Assays. Since immunodiffusion does not detect anti-C5 produced in the primary response, inhibition of C5 hemolytic activity was used as a more sensitive assay. Fig. 2 shows titrations of early and late mouse anti-mouse C5. By plotting percent specific inhibition (calculated from specific lysis) vs. logarithm of antibody
concentrations, we obtained a straight line. The slopes of the lines were comparable for weak and strong antibody, indicating that the average affinities were similar. An extremely strong antiserum will give detectable inhibition at 1/2,500. It has been our experience that any antibody preparation that gives >80% inhibition at 1/25 dilution will also produce a precipitin line in an immunodiffusion assay vs. C5-sufficient mouse serum.

Normal sera from C5-deficient and C5-sufficient strains showed a weak inhibitory activity in the anti-C5 hemolysis assay. This activity was observed even when the C5 in the assay was provided by human serum. At least three normal sera were run with each anti-C5 assay to ascertain the level of anticomplementary activity, which varied from assay to assay. At a dilution of 1/25, anticomplementary activities did not exceed 40% inhibition and were usually less. 50% inhibition was a significant cut-off point for all assays at 1/25. At 1/50, the anticomplementary activity fell to <20% inhibition.

**Cell Transfer to C5-deficient Irradiated Hosts.** To measure directly the capacity to produce anti-C5 of lymphocytes from C5-sufficient mice, we transferred them to C5-deficient irradiated hosts, which would not absorb out the anti-C5 antibodies, but which at the same time would make no response of their own. We therefore ascertained three parameters of the transfer system: (a) the ability of C5-deficient lymphocytes to respond in irradiated hosts; (b) the extent of transfer of C5 antigen and activity caused by transfer of the C5-sufficient immune system; and (c) the inability of the irradiated host to respond on its own to C5.

**Ability of C5-deficient Spleen Cells to Respond in Irradiated Hosts**

**Comparison with C5-sufficient spleen cells.** Fig. 3 shows the primary and secondary responses of C5-deficient irradiated recipients reconstituted with either C5-
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deficient or C5-sufficient nonadherent spleen cells. In this experiment, the first immunization was administered on day 0, the primary response measured on day 14, and the second immunization was also administered on day 14. The secondary response was measured on day 21. The "NSN-PBS" group is a control group of C5-sufficient into C5-deficient chimeras that have been injected with PBS in CFA. Their response is a control for elevation of anticomplementary activity by injection with CFA. Fig. 4 shows an immunodiffusion test of the same antisera shown in the secondary response in Fig. 3. It is apparent that C5-deficient nonadherent spleen cells responded to C5 in this test system. In contrast, C5-sufficient nonadherent spleen cells failed to respond. The C5-sufficient spleen cells do generate some inhibitory activity above and beyond that of normal C5-deficient serum (the shaded area represents the mean inhibitory activity of normal serum plus or minus 2 standard deviations). The small amount of inhibitory activity detected in the serum of C5-sufficient into C5-deficient chimeras appears to be anticomplementary activity generated even by immunization with CFA alone. This experiment has been done four times, with similar results.

In several experiments, the reconstituted C5-deficient hosts were injected with
Inhibition test of tolerant and nontolerant chimeras against B10.D2NSN C5. B10.D2OSN C5-deficient irradiated hosts were reconstituted with either B10.D2OSN (nontolerant) or B10.D2NSN (tolerant) nonadherent spleen cells. The chimeras were immunized with either C5 in CFA (no notation on figure) or PBS-CFA (as labeled NSN-PBS). OSN is OSN into OSN, NSN is NSN into OSN. The first panel is the primary response, measured at a test dilution of 1/25, and the second panel is the secondary response, measured at a test dilution of 1/50. The Y axis is percent inhibition of mouse C5 hemolytic activity. The hatched area is the mean ± 2 SD of normal OSN serum tested in the same assays at the same dilutions.

Immunodiffusion test of tolerant and nontolerant chimeras against B10.D2NSN serum. Mice and experiment are as for Fig. 3, panel 2 (secondary response). Group 1 (1–6), OSN into OSN; group 2 (1–6), NSN into OSN.

Ovalbumin along with the C5 as a specificity control. Table I shows the response to ovalbumin by mice reconstituted with C5-deficient or C5-sufficient nonadherent spleen. The tolerance to C5 was antigen specific, as both populations responded to ovalbumin.

Lack of transfer of C5. C5 is reported to be a product of mouse splenic adherent cells (13). We therefore removed the adherent cells from our spleen cell preparations before transfer to irradiated C5-deficient recipients. The resulting chimeras were assayed for C5 hemolytic activity over the course of several months. Hemolytic C5 levels in C5-deficient irradiated hosts that received nonadherent spleen, whole spleen, or bone marrow of C5-sufficient donors were less than 10 ng/ml at all times.

Despite the lack of evidence of C5 hemolytic activity, the possibility existed that C5
TABLE I
Responses of Tolerant and Nontolerant Chimeras of Ovalbumin

| Donor | Host | Antigens | Response to C5 | Ova |
|-------|------|----------|----------------|-----|
| OSN   | OSN  | C5, Ova  | +              | +(869 ± 323) |
| NSN   | OSN  | C5, Ova  | -              | +(1621 ± 870) |

C5-deficient nonadherent spleen into C5-deficient irradiated host (nontolerant), and C5-sufficient nonadherent spleen into C5-deficient irradiated host (tolerant) chimeras were immunized on the day of reconstitution with mouse C5 and ovalbumin. Ovalbumin titers were assayed on day 14. Indirect titers only are listed. The enhancing reagent was rabbit anti-mouse immunoglobulin at 1/200, absorbed with SRBC. All test sera were preabsorbed with SRBC.

TABLE II
In Vivo Absorption of C5 in C5-deficient Chimeras

| Donor | Antibody transferred | Percent inhibition |
|-------|----------------------|--------------------|
| OSN   | Yes                  | 77.1               |
| NSN   | Yes                  | 78.5               |

C5-deficient B10.D2OSN irradiated hosts were reconstituted with nonadherent spleen from either B10.D2OSN donors or C5-sufficient B10.D2NSN donors. Two of each kind of chimeras were constructed. On day 3 after reconstitution, each mouse was injected with a high titer 50% ammonium sulfate cut of OSN anti-NSN C5. 24 h later, the mice were bled and the sera from the two duplicate chimeras pooled. The inhibition of C5 hemolytic activity at a test dilution of 1/25 was measured.

antigenic activity had nonetheless been transferred. To check for this possibility, we performed an in vivo absorption experiment. Four chimeras were prepared. Two were C5-deficient recipients of nonadherent C5-deficient spleen cells (as C5 deficient controls), and two were C5-deficient recipients of C5-sufficient nonadherent spleen cells. On the 3rd d after transfer, all four mice were injected with a high titer anti-C5 antibody, and the mice were bled the next day. Table II shows the inhibition activities in the sera of these mice. There were no differences in the amounts of anti-C5 detectable in the two pools. We conclude that there was insufficient C5 antigenic activity present in the chimeras to result in absorption of anti-C5 produced in response to immunization.

Inability of irradiated hosts to respond. Unreconstituted lethally irradiated animals eventually die. This death is greatly hastened by the injection of CFA. However, when mice were given 760 rad in a split dose and immunized, some survived for the requisite 14–20 d for a primary response. Unreconstituted mice as well as bone marrow reconstituted mice and surface Ig-negative spleen cell reconstituted mice did not develop a primary response to C5 within 20 d, demonstrating the immunoincompetence of the irradiated host.

Cellular Basis of Tolerance. The results shown in Figs. 3 and 4 indicate that nonadherent spleen of C5-sufficient animals is tolerant with respect to antibody formation to C5. We next determined whether T (nonsurface immunoglobulin-bearing) or B (surface immunoglobulin-bearing) cells are responsible for this tolerance.
Fig. 5 shows a compilation of the data obtained from three experiments in which T cells and B cells from C5-deficient and C5-sufficient mice were transferred into deficient irradiated hosts. The chimeras were immunized with C5 in CFA on day 0.

Recipients of mixed T cells and B cells from the C5-deficient strain showed the highest percentage of responders to C5. A comparable proportion of chimeras reconstituted with C5-deficient T cells and C5-sufficient B cells responded. In contrast, chimeras reconstituted with C5-sufficient T cells and either C5-deficient or sufficient B cells did not respond to C5. From these data, we may conclude that a specific defect in the response to C5 occurs in C5-sufficient T cells, as assayed in the C5-deficient irradiated host. In contrast, B cells of the C5-sufficient strain cooperate with C5-deficient T cells to produce a normal response to C5. Less than 25% of chimeras reconstituted with T or B cells alone responded, indicating that this is a T and B cell-dependent response.

Table III shows the response to ovalbumin of the chimeras in two of the experiments included in Fig. 5. All chimeras reconstituted with both T and B cells from either strain responded to ovalbumin. It further indicates the specificity of tolerance to C5 as well as the T and B cell requirement for the immune response to ovalbumin.

Cell Transfer to Irradiated C5-sufficient Hosts. We next transferred C5-deficient or C5-sufficient nonadherent spleen cells into irradiated C5-sufficient hosts. The recipients were not immunized; instead, their endogenous C5 served as the antigen. The dose of radiation used, which was the same as for the C5-deficient hosts, was not usually lethal for the C5-sufficient hosts.

Fig. 6 shows a plot of the hemolytic C5 levels in irradiated C5-sufficient mice.
TABLE III

Responses of T plus B Reconstituted C5-deficient (OSN) Irradiated Chimeras to Ovalbumin

| T (slg) | B (slg) | Average anti-ovalbumin titer ± SE measured on day 20 or 24 |
|---------|---------|----------------------------------------------------------|
| OSN     | OSN     | 489.7 ± 371                                              |
| ONS     | NSN     | 649 ± 508                                                |
| NSN     | OSN     | 671 ± 557                                                |
| NSN     | NSN     | 403.2 ± 322                                              |
|         | ONS     | 3.5 ± 21                                                 |
|         | NSN     | 2.9 ± 8                                                  |
|         | NSN     | 35.5 ± 101                                               |

Responses to ovalbumin were measured by indirect hemagglutination of ovalbumin-coated SRBC. The enhancing reagent was rabbit anti-mouse immunoglobulin absorbed with SRBC at 1/200. All test antisera were preabsorbed with SRBC. The mice are the same as in Fig. 5.

Fig. 6. Transfer of C5-deficient spleen cells into irradiated C5-sufficient hosts. $80 \times 10^6$ C5-deficient B10.D2OSN nonadherent spleen cells (○) or $80 \times 10^6$ C5-sufficient B10.D2NSN nonadherent spleen cells (■) were transferred into irradiated (760 rad) B10.D2NSN recipients. C5 levels were measured on several days. The Y axis is the percent of normal unirradiated B10.D2NSN C5 levels; the X axis is days after transfer.

Transferred with C5-deficient or C5-sufficient nonadherent spleen cells. One representative experiment out of five is shown. A dramatic drop in hemolytic C5 activity, which starts after day 8, with C5 activity reaching its nadir (10–20% of normal) by day 12, then rising abruptly after day 30, occurs in irradiated C5-sufficient recipients of nonadherent C5-deficient spleen. Recipients of C5-sufficient nonadherent spleen
cells as well as unreconstituted or bone marrow reconstituted chimeras (not shown) show only a minor drop to 70% of normal C5 activity. This drop also occurs at day 12, but the C5 activity returns to normal after about 1 wk. The mechanism of this radiation-induced drop of C5 activity is unknown.

C5 is normally present in serum at concentrations of 50–85 μg/ml. A drop to 20% of normal levels means the elimination or inhibition of at least 40 μg/ml of C5. This is much more C5 than is ever inhibited in anti-C5 assays. In an anti-C5 assay, a strong antiserum will inhibit completely a 1/25,000 dilution of normal mouse serum at an antiserum dilution of 1/250. Thus, such an antiserum at full strength could only inhibit 1% of normal C5 activity. Clearly, a very large inhibitory anti-C5 response would be required to cause such a marked drop in C5 activity, although other mechanisms may be involved. The host cells synthesizing C5 may be affected, as they are known to express C5 on their surface (22).

We next examined the T and B cells for tolerance in the presence of C5. We used our previously described experimental design, except that the cells were transferred into irradiated C5-sufficient hosts. The endogenous C5 served as antigen. In the experiment shown in Table IV, recipients of mixed T and B cells from C5-deficient donors showed a significant drop in C5 activity on both day 12 and day 18. Recipients of C5-sufficient T cells and either C5-deficient or C5-sufficient B cells did not show any drop in C5 activities at any time. Recipients of T cells or B cells alone did not show significant drops in C5 activities.

As both T and B cells are required to produce a drop in C5 activity in these irradiated C5-sufficient recipients, we may conclude that this response is both T and B dependent and so must require antibody. On day 12, either C5-deficient or C5-sufficient donor B cells cooperated with C5-deficient T cells to cause comparable drops in C5 activity. This result confirmed our previous observation of lack of B cell tolerance in the C5-deficient hosts. By day 18, the C5 activity had largely returned to

| Table IV |
| Cellular Basis of Tolerance to C5 in C5-sufficient Hosts (NSN) |
| --- | --- | --- | --- |
| Donor cells | Percent reduction of C5 activity | Day 12 | Day 18 |
| T cells | B cells |  |  |
| OSN | OSN | 38* | 52‡ |
| OSN | NSN | 34§ | 12 |
| NSN | OSN | 0 | 0 |
| NSN | NSN | 0 | 0 |
| OSN | — | 11 | 9 |
| NSN | — | 0 | 0 |

C5-sufficient B10.D2NSN irradiated hosts (760 rad) were reconstituted with either 35 × 10⁶ slg⁺ (B) cells or 28 × 10⁶ slg⁻ (T) cells or both from C5-sufficient or C5-deficient strains, as illustrated in the table. Hemolytic C5 activity was measured 12 and 18 d after cell transfer. The percent reduction in C5 activity below that of unreconstituted controls is listed. The P values refer to the comparison between the indicated groups and the unreconstituted control as compared by Student’s t test. No other groups were statistically different from the controls.

* 0.010 < P < 0.025.
§ 0.001 < P < 0.005.
‡ 0.025 < P < 0.030.
normal in the recipients of C5-sufficient B cells and C5-deficient T cells, whereas C5 activities continued to fall in recipients of C5-deficient T and B cells. As predicted from the experiments in C5-deficient hosts, recipients of T cells from C5-sufficient donors were tolerant.

**Maintenance of Tolerance.** The transfer into C5-deficient host system also allows us to examine the question of maintenance of tolerance. How long after transfer does the tolerance last, and what is the influence of externally supplied antigen on the duration of tolerance?

Table V shows the timing of the loss of tolerance in irradiated C5-deficient hosts reconstituted with C5-sufficient nonadherent spleen cells. It is seen that by 28 d after reconstitution, two immunizations with C5 resulted in 70% of chimeras that responded to C5. Primary responses to immunization at day 28 were, in general, poor, both in recipients of C5-sufficient or C5-deficient cells.

The basis for the loss of tolerance by day 28 is unknown. Two possibilities exist. The C5-deficient host could have recovered, or the C5-sufficient reconstituting cells could simply have lost tolerance in the absence of antigen. We have results suggesting that the first possibility is correct. Table VI shows the survival figures for mice reconstituted either with $5 \times 10^5$ anti-Thy-1.2-treated bone marrow cells or nonadherent C5-sufficient spleen, immunized twice starting on day 28 after reconstitution. Although the bone marrow dose was not sufficient to reconstitute most of the mice, many of the surviving mice responded to C5. As shown by the average percent inhibition of C5, many responded better than mice reconstituted with C5-sufficient

### Table V

**Loss of Tolerance in C5-sufficient (NSN) into C5-deficient (OSN) Irradiated Host Chimeras**

| Day of first immunization | OSN → OSN | OSN → OSN |
|--------------------------|-----------|-----------|
| 0                        | 100%      | 100%      |
| 12                       | 100%      |           |
| 28                       | 100%      |           |
| NSN → OSN                | 0%        | 0%        |
|                          | 60-80%    |           |

C5-sufficient nonadherent spleen into C5-deficient irradiated host chimeras were immunized at various times after reconstitution. The percent of mice responding >50% inhibition after two immunizations is given. Data for C5-deficient nonadherent spleen into C5-deficient irradiated hosts are given for comparison.

### Table VI

**Host Response after 28 d**

| Donor                | Host | Survival (day 49) | Average percent inhibition of C5 |
|----------------------|------|-------------------|----------------------------------|
| OSN bone marrow      | OSN  | 7/28              | 54.1 ± 26.58                     |
| NSN spleen           | OSN  | 6/9               | 23.37 ± 1.46                     |

C5-deficient irradiated hosts were reconstituted with either $70 \times 10^6$ B10.D2NSN C5-sufficient nonadherent spleen cells or $5 \times 10^6$ B10.D2OSN anti-Thy-1.2 plus complement-treated bone marrow (minimum reconstitution) after immunization on day 28 and day 42.
spleen cells, which responded poorly in this instance. Because the bone marrow recipients respond, we suspect that the irradiated host recovers before day 28, causing a response to C5.

Whatever the cause of the loss of tolerance, the question of maintenance of tolerance by antigen is still important. Table VII shows the results of an experiment in which the C5-sufficient donor cells were transferred to C5-deficient irradiated recipients and the chimeras given an immunizing dose of C5 at various times before day 28. For comparison, results with C5-deficient donor cells are also given. These times were day 10 (pre-immunized NSN donors), day 0, and day 12 (in the C5-deficient host). The immunizations on day 0 or on day 12 maintained tolerance, such that two immunizations starting on day 28 did not result in a response to C5. Mice immunized starting on day 0 or day 12 and continuously immunized every 2 wk thereafter also remained tolerant for several months (results not shown). These results indicate that the presence of antigen is required to maintain tolerance. They also show that antigen in CFA maintains tolerance.

**Discussion**

We examined natural immunologic tolerance to an autologous soluble antigen in congenic strains of mice characterized by either the presence or the absence of C5. The two main findings were that tolerance was mediated by T cells and that the antigen was necessary to maintain tolerance. Before discussing the implications of these observations, it is essential to recall some of the unique features of this experimental model because they have a direct bearing on the interpretation of the data. A number of reasons make this system particularly relevant for the study of self tolerance. First, the antigen C5 is well characterized, a two chain disulfide linked glycoprotein with a serum concentration of 50-85 μg/ml and a 206,000 mol wt, slightly larger than immunoglobulin, the tolerogen par excellence. Second, highly sensitive assays were developed to determine the presence of C5 and anti-C5 by hemolytic activity and inhibition of same. These assays detected minute quantities of antigen after cell transfer and could monitor the change of serum C5 activity in irradiated C5-sufficient chimeras. Third, B10.D2OSN (C5 deficient) and B10.D2NSN (C5 sufficient) strains of mice, because they are congenic, permit us to examine not

### Table VII

**Maintenance of Tolerance by Antigen in CFA**

| Donor  | Day of immunization | Percent responding at day 49 |
|--------|---------------------|-------------------------------|
|        | 0 | 12 | 28 ± 42 |                               |
| NSN    | — | —  | Yes     | 72                            |
| NSN    | — | Yes | Yes     | 0                             |
| NSN    | Yes | —  | Yes     | 16.7                          |
| Preimmunized | — | Yes | —       | 83                            |
| NSN    | — | —  | Yes     | 100                           |

C5-sufficient into C5-deficient irradiated host chimeras were immunized with mouse C5 at various times before and during the 1st mo after reconstitution. The preimmunized donors were immunized with mouse C5 10 d before killing, with the response measured on day 49. The percent of mice responding above 50% at a test dilution of 1/25 are given.
only the immunocompetence of the lymphoid cells in irradiated hosts of either strain, without graft-vs.-host or host-vs.-graft reaction, but also whether cells of putatively tolerant animals were unresponsive either in the presence of the antigen in its native form or, more importantly, in its absence.

A suitable positive control was provided by irradiated C5-deficient hosts repopulated with syngeneic nonadherent spleen cells and challenged with C5 in CFA. Both cell repopulation and immunization were necessary to produce anti-C5 antibody detectable by the hemolytic inhibition assay. In the secondary response, anti-C5 antibody activity was also detectable by immunodiffusion. In contrast, C5-deficient irradiated recipients of C5-sufficient nonadherent spleen cells failed to produce anti-C5 antibody activity both in the primary and the secondary response. Because this tolerance can be adoptively transferred by lymphoid cells into irradiated congenic hosts and does not affect the response to ovalbumin, it is antigen specific. Further, this tolerance represents central unresponsiveness, as no antigen carryover could be detected either by hemolytic assay or in vivo absorption.

The model further demonstrated that tolerance to C5 was mediated by T cells. This was observed both in irradiated C5-sufficient and C5-deficient hosts repopulated with mixtures of T and B cells. The finding that T cells were tolerant to a soluble native antigen is consistent with previous data (1-3, 23) and confirmed the observation that only one cell type needs to be unresponsive for an animal to be tolerant to a T-cell-dependent antigen (4). In this paper, we have not examined which subsets of T cells are unresponsive. For example, it is unknown whether C5 renders helper T cells unresponsive directly or whether unresponsiveness is mediated by suppressor T cells or their soluble products. Alternatively, both suppressor T cells and helper T cells specifically tolerant to C5 might be present, but the former are not necessarily related to the latter. In support of this view is the observation that two different forms of the same antigen can elicit either suppressor T cells or tolerance (24) and that there is a dichotomy in the cellular mechanism of these two distinct forms of unresponsiveness (25).

The fact that B cells were not tolerant to C5 is perhaps more intriguing, not only because it raises questions about the susceptibility of tolerance in T-vs.-B cells to autologous antigens, but also because it challenges the clonal abortion hypothesis, which postulates that B cells are irrevocably tolerized by self antigens early in their maturation. B cells might be responsive either because of the nature of the antigen or because of insufficient dose. Whereas antigens with multiple epitopes are particularly effective for B cell tolerance (26), there is no known soluble antigen that is able to render T but not B cells tolerant because of its structure only. The serum concentration of C5 (at 50 µg/ml) might be insufficient to induce B cell tolerance, though this concentration is known to induce B cell tolerance to heterologous gammaglobulin either in vitro (27, 28) or in utero (29). Because it is known that neonatal cells are more susceptible than adult cells to tolerance induction (30) and because the C5-sufficient mice had been exposed neonatally to C5, it is unlikely that the lack of B cell tolerance is due to the level of tolerogen. In autoimmunity, B cells are known to respond to autoantigens (31). Mitogens also, such as lipopolysaccharides, can stimulate B cells to produce autoantibody (8). Nonetheless, these findings are difficult to extend to physiologic B cell responsiveness to native soluble antigens of relatively high serum concentration. Furthermore, as mentioned above, the presence of the antigen in the
host complicates the issue. For example, studies on the specificities of antibodies to hemoglobin (32), cytochrome (33), and insulin (34) have been done, but in these studies the extent to which antibody is absorbed by native antigens was ignored. The transfer of spleen cells into irradiated C5-sufficient mice allows us to observe tolerance in the presence of the native antigen in its original distribution. When C5-deficient spleen cells are transferred, a massive drop in C5 level occurs. In contrast, sufficient spleen cells do not mediate this drop. Because the drop in C5 levels required the participation of both T and B cells, it suggests that it is antibody dependent. This is the only system using normal cells in which tolerance in the presence of the antigen may be reliably observed.

One of the leading theories of natural tolerance is the clonal deletion hypothesis (35, 36), further refined to clonal abortion (37). This theory postulates that B cells, during differentiation, while they acquire immunoglobulin on their surface, pass through a stage in which they are particularly susceptible to tolerance induction. As a consequence, immunoglobulin-bearing B cells able to react to tolerogen should be absent. The finding that mature C5-sufficient splenic cells separated by virtue of surface immunoglobulin respond to C5 is in direct contradiction with the clonal abortion hypothesis. This argument is further strengthened by our preliminary results that suggest that B cells are not tolerant, even in the C5-sufficient irradiated host. But the clonal abortion hypothesis, which might apply to acquired unresponsiveness of antigen with repeated epitopes, has not been tested for a natural antigen. Some even have questioned whether B cell tolerance to natural antigen exists (38). On the other hand, it is conceivable that a subset of B cells might be tolerant to C5, but unresponsiveness in this subset is masked by the response of the large proportion of mature B cells. But whether physical or functional deletion occur, as proposed in clonal anergy (39), might be difficult to prove or disprove.

Finally, the role of the antigen in the maintenance of unresponsiveness should be emphasized in this physiological model of tolerance. The form of antigen required and the duration of tolerance are both of interest. In C5-deficient irradiated mice reconstituted with C5-sufficient spleen cells, tolerance spontaneously wanes after 1 mo. It is unclear whether tolerance was broken or spontaneously waned, but we favor the latter interpretation. When C5-deficient irradiated hosts were repopulated with a minimum dose of bone marrow cells, insufficient to enable the host to respond to C5 when they had been immunized immediately after cell reconstitution, they form anti-C5 antibody when challenged 1 mo later, suggesting that we are dealing not with a donor, but rather a host-immune response. On the other hand, it is clear that administration of C5 in CFA, either on the day of cell transfer or 12 d later, maintains unresponsiveness. In addition, repeated injections of C5 in CFA maintained unresponsiveness for several months. Although the cellular mechanism of the maintenance of tolerance is unknown, it seems paradoxical that antigen in CFA, the immunogenic form, is, in fact, tolerogenic. In contrast, C5 in its native form in the C5-sufficient host can be immunogenic. This paradox suggests that in this natural model of tolerance, the form of the antigen is not the direct determinant of the type of response by the immune system. It shows that the antigen is necessary not only to induce tolerance but also to maintain it. Thus, natural tolerance to C5 appears to be an active process, dependent upon both the presence of the antigen as well as T cells. In contrast,
capacity to produce autoantibody by B cells from tolerant or nontolerant hosts in the presence or absence of antigen appears to remain unimpaired.

Summary

A unique experimental model is described, where natural immunologic tolerance to a well-defined soluble native antigen (murine C5) is examined in congenic strains of mice that differ only by the presence or the absence of C5. A highly sensitive hemolytic assay was developed to detect nanogram amounts of C5 as well as an assay of anti-C5 inhibition of C5 hemolytic activity. The latter was more sensitive than immunodiffusion. Two reciprocal approaches were used to study the cellular basis of tolerance in irradiated hosts of either strain. In the first, lymphoid cells from either strain were transferred to irradiated B10.D2OSN hosts that were lacking C5 and so would not hinder detection of anti-C5 antibody upon challenge with murine C5. Second, lymphoid cells from either strain were transferred to irradiated B10.D2NSN hosts, whose native C5 provided the antigenic stimulus. The immune response of whole nonadherent spleen cell suspension as well as mixtures of T and B cells (separated on the basis of surface immunoglobulin) from either strain were studied. In addition, the duration of tolerance and the antigen requirement to maintain it in irradiated C5-deficient hosts repopulated with C5-sufficient spleen cells was examined.

The positive control of irradiated C5-deficient hosts repopulated with syngeneic spleen cells showed a primary and secondary response to immunization. In contrast, C5-sufficient spleen cells failed to respond both in the primary and the secondary response. Because the unresponsiveness was not caused by antigen carryover and was not antigen specific, it represents central tolerance. In C5-sufficient irradiated hosts (where immunization was not required and antigen was present in natural form and physiological concentration), transfer of C5-deficient cells mediated a drop in C5 levels to 10–20% of that noted in unreconstituted controls. T and B cell mixing experiments from the two strains into deficient or sufficient hosts demonstrated that tolerance is T cell dependent and that C5-sufficient or -deficient B cells could cooperate with nontolerant C5-sufficient T cells to produce significant anti-C5 antibody or mediate a significant drop in C5 levels. In addition, the presence of antigen was necessary to maintain tolerance.

In conclusion, these results show that (a) natural tolerance to C5 is an active process that is T cell dependent and requires the presence of antigen; (b) in this natural model, clonal abortion does not seem to occur; and (c) both tolerant and nontolerant B cells retain the capacity to produce autoantibody.

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