CELL-TO-CELL INTERACTION IN THE IMMUNE RESPONSE

X. T-Cell-Dependent Suppression in Tolerant Mice*

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A substantial literature has appeared in the last decade on immunological tolerance (1–4). The demonstration that optimal antibody production to a variety of antigens involves at least two cell types (T cells and B cells) has necessitated reassessment of the concept of tolerance. One of the major issues has been to define the site of tolerance induction in a given experimental situation; in other words whether T cells, B cells or both have been rendered unresponsive (4, 5). There is now clear evidence for specific unresponsiveness in the B-cell lineage (3, 6, 7). When induced by injection of a haptenic determinant coupled to a nonimmunogenic carrier (6, 7) it is stable on adoptive transfer. The decrease in the number of cells binding labeled tolerogen observed by some investigators (6, 8) implies that tolerance in this situation reflects deletion of a specific clone of antigen-reactive (B) cells. Unresponsiveness has in addition been demonstrated at the level of the T cell (reviewed in 8, 9). This form of tolerance in contrast to B-cell tolerance is associated with normal or even increased numbers of antigen-binding cells. Furthermore it may, in certain situations, be "infectious"; thus cells from tolerant donors have the capacity to inhibit antibody production by nontolerant hosts (10) or by normal cells (11). The implication of these findings is that tolerance of this kind is due, not to deletion of antigen-sensitive cells, but to a positive suppressor effect by tolerant cells, presumably thymus-derived (12, 13). To date most of the published evidence for "suppressor" T cells is indirect. Two examples in particular are worth citing. First, depletion of T cells by thymectomy or antilymphocyte serum treatment (14) has under certain conditions resulted in significant enhancement of subsequent (IgM) antibody production to "thymus-

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independent" antigens. The effect however was not readily reversible in all cases by addition of nontolerant T cells, nor was it shown to be specific. Second, thymus cells have been found by some groups (15), but not by others (reviewed in 16), to suppress the adoptive immune response to the "thymus-independent" antigen, pneumococcal polysaccharide type III.

The data given in the present paper provide further evidence for the existence in tolerant mice of T cells which exert an active suppressor effect on the production of antibody, particularly of the IgG class.

Materials and Methods

Animals.—Male and female mice of the inbred CBA/H/WEHI, AKR/J, and CBA/J strains were used. The latter two strains were obtained from the Jackson Laboratories, Bar Harbor, Maine, and the former from the Walter and Eliza Hall Institute, Melbourne. Their pedigree and maintenance have been described previously (17).

Cell Suspensions.—Single cell suspensions were obtained from the spleen and thoracic duct lymph by methods outlined in a previous communication (17). They were injected intravenously in cell transfer experiments.

Antigens.—Sheep erythrocytes (SRBC)[1] horse erythrocytes (HRBC), and fowl immunoglobulin G (FγG) were obtained as outlined before (17). Donkey erythrocytes (DRBC) were supplied by the C.S.I.R.O. Division of Animal Health, Sydney, by courtesy of Dr. J. Dineen.

Injections.—Cell suspensions and antigens were injected intravenously or intraperitoneally as indicated in the text.

Operative Procedures.—Thoracic duct cannulation was carried out as described previously (17).

Irradiation.—Details of the technique have been outlined in a previous paper of this series (17). Recipients received a total body dose of 750–800 R on a Phillips RT250 X-ray machine. Cell suspensions were injected within 3 h of irradiation and antigens on the same day. All irradiated mice were given polymyxin B (100,000 IU/liter) and neomycin (10 mg/liter) in their drinking water.

Immunization.—For priming to heterologous erythrocytes (SRBC, DRBC, HRBC), 5×10^8 cells were injected intravenously. Mice were immunized to protein antigens by giving 500 μg of alum-precipitated antigens and 2×10^9 killed pertussis organisms. Alum precipitation was carried out as previously described (17). Cells from such donors were used 4–8 wk postpriming. Subsequent challenge or booster injections involved intraperitoneal inoculation with 100 μg of fluid antigen.

Tolerance Induction.—A state of unresponsiveness to FγG was induced by one of two methods. In the first, FγG was ultracentrifuged at 100,000 *g* for 2½ h to remove aggregated protein and the top one third carefully removed. Before centrifugation each batch of FγG was tested for antimouse leukocyte agglutinins and absorbed if necessary with $\frac{1}{10}$th vol of thymus cells for 3 h at 4°C. CBA mice were injected intraperitoneally with 15 mg of deaggregated material immediately after ultracentrifugation. Thoracic duct lymphocytes (TDL) and spleen cells were collected 6–12 days later since preliminary experiments had indicated that donors were tolerant at that time. The second method of tolerance induction utilized the cyclophosphamide technique (18). Each mouse received an intraperitoneal injection of 5 mg alum-precipitated FγG (FγGA) and 2×10^9 killed pertussis organisms, followed the next day by a

[1] *Abbreviations used in this paper*: DRBC, donkey erythrocytes; FBS, fetal bovine serum; FγG, fowl immunoglobulin G; FγGA, alum precipitated FγG; HRBC, horse erythrocytes; PFC, plaque-forming cells; 19S PFC, direct plaque-forming cells; 7S PFC, indirect (developed) plaque-forming cells; SRBC, sheep erythrocytes; TDL, thoracic duct lymphocytes.

subcutaneous injection of cyclophosphamide at a dose of 1 mg/10 g body weight. Cells for experimental purposes were collected 2 wk later.

Preparation of anti-θ C3H Serum.—Anti-θ C3H serum was raised in AKR/J mice as described previously (17). After inactivation at 56°C for 30 min the potency of each batch was tested by cytotoxicity against CBA thymus cells and TDL or lymph node cells. Agarose-absorbed guinea pig serum was used as the source of complement. For preparation of T-cell depleted cell populations, 1 ml of anti-θ serum was added to 10^8 spleen cells at a concentration of 25×10^6 cells/ml. After incubation for 30 min at 37°C, the cells were washed twice and exposed (5×10^7 cells/ml) to complement diluted 1 in 6 for a further period of 30 min at 37°C.

Separation of T Lymphocytes.—Lymphoid cell suspensions enriched for T lymphocytes were obtained by passing normal CBA spleen cells through columns according to the method of Basten et al. (19) or Campbell and Grey (20). The efficiency of separation was tested by treating the final cell suspension with radioiodinated immune complexes (21) to estimate the number of residual B cells. Experiments in which suspensions contained more than 5% contaminating B cells were discarded.

Preparation of Iodinated Antigen.—FγG was iodinated with ^{125}I (Radiochemical Centre, Amersham, England, cat. no. IMS3) according to the method of Byrt and Ada (22). The specific activity was approximately 25 μCi/μg for antigen binding studies and 1,000 μCi/μg for "suicide" experiments. Before iodination FγG was absorbed extesnsively against mouse lymphocytes to remove nonspecific antimouse leukocyte agglutinins (21). When FγG was being used to detect possible antigen carryover it was labeled with ^{125}I *before* ultracentrifugation to circumvent the problem of spontaneous reaggregation during the iodination procedure.

Preparation of Iodinated Rabbit Antimouse IgG.—A polyvalent rabbit antimouse IgG was obtained from Dr. Noel Warner. Its method of preparation was described previously (23). Iodination was carried out according to the technique described above except that the specific activity was approximately 12–15 μCi/μg.

Radioactive Antigen Suicide Technique.—Spleen cells from FγG-tolerant donors were treated with highly substituted [^{125}I]FγG at a concentration of 10 μg/10^8 cells. After incubation at 37°C for 60 min, excess antigen was removed by centrifugation through fetal bovine serum (FBS) gradients and the cell pellet resuspended in RPMI 1640 medium (Microbiological Associates, Inc., Bethesda, Md.). The cells were then incubated overnight at 4°C, recounted and injected intravenously into irradiated hosts. Control cell populations were treated with [^{127}I]FγG under identical conditions.

Detection of Antibody-Forming Cells.—Antibody-forming cells were detected as described before (17). Various target cells were used: (*a*) HBRC or DRBC to detect anti-HRBC or anti-DRBC plaque-forming cells (PFC) respectively; (*b*) FγG-coated SRC to detect anti-FγG PFC.

Serum Antibody Titers.—To FγG were determined by hemagglutination of FγG-coated SRBC made up to a 2% solution in saline.

Detection of Antigen-Binding Cells.—The number of antigen-binding cells in TDL and spleen cell suspensions was estimated by a radioautographic technique. 400 ng of [^{125}I]FγG was added to aliquots of 10^7 cells suspended in 0.4 ml Eisen's solution containing 5% FBS, and 1.5 mM sodium azide. The suspension was incubated for 30 min at 4°C, washed free of excess antigen, smeared and prepared for radioautography in the standard manner. Only cells carrying 10 grains or more were scored as positive. In some experiments the integrity of the receptors on lymphocytes from tolerant mice was examined by incubating them with [^{125}I]FγG at 37°C for 60 min in RPMI 1640 with 10% FBS in the absence of sodium azide. Under these circumstances it was possible to assess whether the cells retained the capacity to show receptor motility and cap formation (24).

Trypsin Treatment.—Twice crystallised trypsin (Sigma Chemical Co., St. Louis, Mo., cat. no. TA253) was dissolved in Hepes-buffered Eagle's medium at a concentration of 0.1–1 mg/ml. Spleen cells ($2–3 \times 10^7$ cells/ml) from normal and tolerant donors were incubated in the

trypsin solution for 10–15 min at 37°C. The reaction was then stopped by addition of excess FBS and the cells washed by centrifugation through FBS gradients. The efficiency of enzyme treatment was assessed radioautographically in each experiment. Aliquots of treated and control cells were incubated with ^{125}I-labeled rabbit antimouse IgG (vide supra) and the number of cells with residual immunoglobulin receptors estimated. The experimental results were used if less than 5% of the treated final cell suspension were still found to carry receptors.

Viability Counts.—Cell viability was estimated by the dye exclusion technique, as routinely done in these laboratories (17).

Removal of Dead Cells.—Dead cells were removed from suspensions by the method of von Boehmer and Shortman (25). The procedure was required only when spleen cells were being used, as TDL were usually 95–100% viable.

Statistical Analyses.—These were performed as described in a previous paper in this series (17).

RESULTS

Tolerance to Deaggregated FγG.—In preliminary experiments tolerance to FγG was induced in CBA/J and CBA/H/WEHI strains of mice by injecting a dose of 15 mg deaggregated material per mouse. Tolerance was demonstrable between 6 and 10 days after inoculation of the deaggregated material. It was stable on adoptive transfer to irradiated mice as illustrated in Tables I and II and predominantly affected IgG production (as measured by indirect PFC) rather than IgM (direct PFC). Furthermore, the response of the same recipients of tolerant cells to an unrelated antigen, DRBC or HRBC, was unimpaired; this established the specificity of tolerance. In all subsequent transfer experiments the existence of tolerance was checked by comparing the adoptive responses of normal and tolerant cells to FγG and to a control antigen.

Effect of Trypsinization on the Tolerant State.—The stability of the system was further tested by preincubating tolerant spleen cells with trypsin before adoptive transfer. As shown in Table I, enzyme treatment failed to abrogate unresponsiveness. The efficacy of trypsinization was established in each experi-

TABLE I

Stability of FγG Tolerance in Adoptive Transfer: Irreversibility after Pretreatment with Trypsin

Group	No. and source of cells given	No. of irradiated recipients*	PFC/spleen at 7 days‡			
			Anti-FγG		Anti-DRBC	
			19S	7S	19S	7S
1	3×10^7 FγG-tolerant spleen	8	65(115–35)§	120(265–55)	845(1,805–395)	8,470(13,300–5,395)
2	3×10^7 FγG-tolerant spleen cells pretreated with trypsin	7	535(895–320)	165(265–55)	1,580(2,730–915)	5,985(9,455–3,460)
3	3×10^7 normal spleen	7	395(640–140)	3,835(4,815–2,990)	1,495(2,575–840)	5,270(7,260–3,760)

P values between groups—7S PFC to FγG: group 1 cf. 2, NS; 1 cf. 3, <0.01. Although, in this experiment, trypsin pretreatment significantly increased the 19S PFC response to FγG ($P < 0.05$), this was not observed in other experiments.

* Each recipient was given 500 μg FγGA and 5×10^8 DRBC.

‡ Data pooled from two experiments.

§ Geometric mean, upper and lower limits of SE.

TABLE II

Capacity of FγG Tolerant Spleen Cells to Suppress the Primary Adoptive Response to FγG: Dependence of the Suppressor Effect on a θ-Sensitive Cell Population

Group	No. and source of cells given	No. of irradiated recipients*	PFC/spleen at 7 days‡			
			Anti-FγG		Anti-DRBC	
			19S	7S	19S	7S
1	1.5×10^7 FγG-tolerant spleen cells	7	460(895–235)§	105(255–45)	1,250(1,700–920)	3,210(3,690–2,790)
2	1.5×10^7 FγG-tolerant spleen cells + 1.5×10^7 normal cells	8	185(510–65)	30(60–15)	1,680(2,485–1,135)	6,025(9,050–4,300)
3	1.5×10^7 FγG-tolerant spleen cells treated with anti-θ serum + 1.5×10^7 normal spleen cells	8	240(435–130)	4,645(5,530–3,905)	2,035(2,320–1,445)	7,390(9,890–5,430)
4	1.5×10^7 normal spleen cells	7	190(460–80)	1,195(1,545–930)	245(390–155)	3,500(4,335–2,820)
5	3×10^7 normal spleen cells	9	245(460–130)	2,525(3,050–760)	2,430(2,675–2,210)	7,330(8,500–6,315)
6	3×10^7 tolerant spleen cells	8	495(1,050–235)	150(330–70)	925(1,305–655)	5,595(6,375–4,910)

P values between groups—19S PFC to FγG: group 1 cf. 2, NS; 2 cf. 3, NS; 1 cf. 4, NS; 2 cf. 5, NS; 5 cf. 6, NS. 7S to FγG: 1 cf. 2, NS; 2 cf. 3, <0.01; 3 cf. 5, = 0.05; 5 cf. 6, <0.05.

* Each recipient was given 500 μg FγGA and 5×10^8 DRBC.

‡ Data pooled from two experiments.

§ Geometric mean, upper and lower limits of SE.

ment by testing for residual surface immunoglobulin with ^{125}I-labeled rabbit antimouse IgG: less than 5% of the treated cells still bound the radioactive anti-immunoglobulin. The normal response of DRBC (group 2) indicated that the trypsin-treated cells had not been damaged nonspecifically and opsonized, but were indeed homing to the spleen. Trace labeling studies with ^{51}Cr were therefore considered unnecessary.

Antigen Carryover in Adoptive Transfer.—The problem of antigen carryover was examined by injecting ^{125}I-labeled deaggregated FγG intravenously into tolerant donors either at the time of tolerization, or 24 h before death, i.e., 6–10 days after tolerization. The former group received 100 μg [^{125}I]FγG and the latter 20 μg [^{125}I]FγG, which had been ultracentrifuged after iodination. It is theoretically possible that ultracentrifugation selected against labeled antigen. This, however, is unlikely since the substitution ratio was 1–2 iodine atoms/molecule FγG. Mice were killed by exsanguination and the amount of residual radioactivity measured in blood and spleen. Although iodide was detectable in the blood, samples containing 3×10^7 washed spleen cells had counts which were never significantly above background. The likelihood of transfer of appreciable amounts of non-cell-associated toleragen was therefore considered minimal.

Effect of Tolerant Spleen Cells on the Adoptive Response of Normal Spleen

Cells.—Spleen cells from tolerant and normal mice were transferred alone or in combination into irradiated recipients together with FγG in immunogeneic form and a control antigen, DRBC. As shown in Table II, normal spleen cells alone (groups 4, 5) produced an appreciable indirect (7S) PFC response to FγG and DRC on day 7. When 1.5×10^7 cells from tolerant animals were added, however, a significant decrease ($P < 0.01$) in indirect FγG PFC but not in DRBC PFC was observed (group 2). No significant reduction in direct (19S) PFC occurred. The inhibitory effect of the tolerant spleen cells was reversible by pretreatment with anti-θ C3H serum and complement (group 3). This strongly suggested that tolerance in this situation was determined by a positive "suppressor" action on the part of anti-θ serum-sensitive (T) cells.

Effect of Antigen Suicide on the Inhibitory Action of FγG-Tolerant Cells.—It was decided to examine whether the inhibitory effect of tolerant spleen cells could be abrogated by pretreatment with [^{125}I]FγG under conditions known to "suicide" T cells (as well as B cells). Spleen cells from tolerant mice were incubated with either [^{125}I]FγG or [^{127}I]FγG and transferred together with normal spleen cells into heavily irradiated hosts. As shown in Table III, [^{125}I]FγG-treated spleen cells failed to suppress a primary adoptive response of normal

TABLE III

Abrogation of the Suppressor Effect of FγG-Tolerant Spleen Cells by [^{125}I]FγG

Group	No. and type of cells given	No. of irradiated recipients*	7S PFC/spleen at 7 days	
			Anti-FγG	Anti-HRBC
1	1.5×10^7 tolerant spleen cells preincubated with [^{127}I]FγG	7	190 (500–70)‡	20,950 (22,880–19,190)
2	1.5×10^7 normal spleen cells	7	9,000 (9,800–8,280)	19,950 (21,680–18,360)
3	1.5×10^7 normal spleen cells + 1.5×10^7 tolerant spleen cells preincubated with [^{127}I]-FγG	8	720 (870–590)	59,760 (63,910–55,870)
4	1.5×10^7 normal spleen cells + 1.5×10^7 tolerant spleen cells preincubated with [^{125}I]-FγG	7	10,660 (11,990–9,470)	62,460 (68,040–57,330)
5	1.5×10^7 tolerant spleen cells preincubated with [^{125}I]FγG	6	830 (1,070–650)	18,190 (20,300–16,310)

P values between groups—7S PFC to FγG: group 1 cf. 2, <0.01; 1 cf. 3, NS; 2 cf. 3, <0.001; 3 cf. 4, <0.001; 2 cf. 4, NS.

* Each recipient was given 500 μg FγGA and 5×10^8 HRBC.

‡ Geometric mean, upper and lower limits of SE.

spleen cells to FγG (group 4) whereas the control ([^{127}I]FγG-treated) cells did so as usual (group 3); ^{125}I-treated-tolerant cells injected alone were ineffective in transferring an FγG response although they were capable of inducing normal antibody production to the control antigen, HRBC. These findings implied that the "suppressor" T cells in tolerant spleen (*a*) were able to bind antigen specifically, and (*b*) were radiosensitive.

Duration of the Suppressor Effect of Tolerant Spleen Cells on the Adoptive Primary Response to Normal Spleen Cells.—In the above experiments, numbers of PFC were assayed at 7 days after adoptive transfer. To study the duration of suppression on transfer, the following experiment was performed: spleen cells from normal donors were transferred alone or in combination with tolerant cells into irradiated hosts together with FγG and HRBC. Groups of recipients were killed at days 4, 7, 10, and 15. On each occasion spleens were collected for PFC assay and blood was obtained for serum antibody levels. As shown in Figs. 1 and 2, the inhibitory effect of tolerant spleen cells (*a*) was observed with indirect, not with direct PFC, and (*b*) persisted, in the case of indirect PFC, being more pronounced at days 10 and 15 than at day 7. In this particular experiment, the difference in 7S PFC response to FγG was statistically significant at days 10 and 15, not at day 7. The serum antibody levels displayed a trend similar to the levels observed for indirect PFC (Fig. 3).

Restoration of the Capacity of Anti-θ Serum-Treated Tolerant Spleen Cells to Transfer an Adoptive Primary Response to FγG by Addition of Normal T Cells.—To determine whether tolerance operated also at the B-cell level, tolerant spleen cells were treated with anti-θ serum and adoptively transferred together with antigen and a purified population of normal T cells. As shown in group 5 of Table IV, it is evident that, in this situation, normal antibody responses were obtained. Thus the B cells in the tolerant population were responsive to FγG, provided the T cells present were removed and replaced by normal T cells.

Antigen-Binding Cells.—The above results implied that B cells reactive to FγG had not been deleted from the tolerant cell population. Since conventional antigen-binding techniques are now considered to detect B rather than T cells, the presence of specifically-reactive B cells was assessed directly by comparing the numbers of [^{125}I]FγG labeled cells in tolerant spleen and TDL with those from normal or primed mice. No reduction in antigen-binding cells was found in lymphocytes from tolerant mice (Table V). Indeed their frequency fell between that of normal and primed controls. In other studies, the receptors on tolerant cells underwent "cap" formation in the usual manner; this implied that at least this stage in interaction with antigen was unimpaired.

Transfer of Tolerance to Normal Recipients.—The capacity of FγG-tolerant spleen cells to suppress an adoptive primary response to the same antigen (Tables II and IV) implied that it should be possible to induce tolerance in a normal recipient by inoculation of syngeneic tolerant cells. Initial attempts by transfer of 5×10^7 viable cells were unsuccessful. When, however, the number

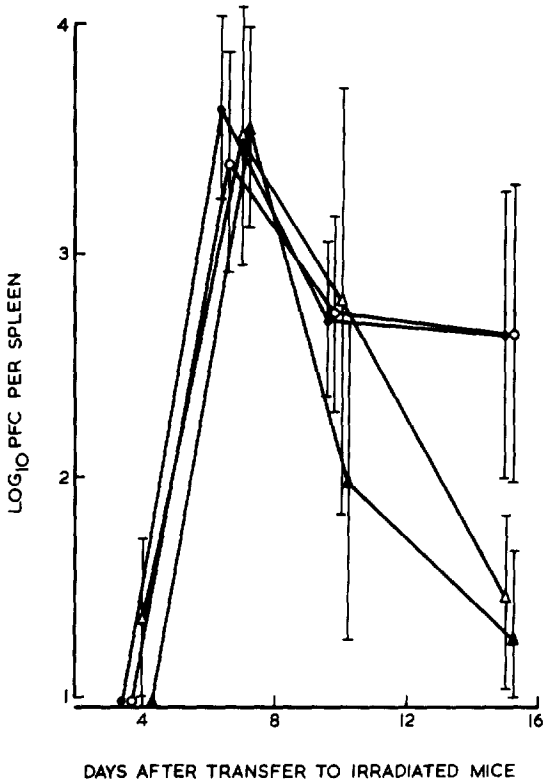

FIG. 1. Time response curve of the inhibitory effect of tolerant spleen cells on the primary adoptive 19S PFC response to FγG in irradiated hosts. 15×10^6 spleen cells from normal mice were given intravenously alone or together with 15×10^6 spleen cells from FγG-tolerant mice. Irradiated recipients were challenged with 500 μg FγG A intraperitoneally and 5×10^8 HRBC intravenously. △, 19S anti-FγG PFC in recipients of normal spleen cells; ▲, 19S anti-FγG PFC in recipients of normal and FγG-tolerant spleen cells; the differences between these two groups are not significant. ○, 19S anti-HRBC PFC in recipients of normal spleen cells; ●, 19S anti-HRBC PFC in recipients of normal and FγG-tolerant spleen cells; the differences between these two groups are not significant. Seven–eight mice per group. The vertical bars represent the 95% confidence limits.

of tolerant spleen cells was increased to at least 25×10^7 cells per recipient a significant reduction in 7S FγG PFC was observed (Table VI) even after recipients had received a second injection of FγG. Recipients of spleen cells from nontolerant donors or from donors rendered unresponsive by a cyclophosphamide regime, known to delete antigen-sensitive T cells (26), developed a normal response to FγG as well as to the control antigen, DRBC.

The need to use large numbers of cells in these studies made it particularly important to exclude antigen carryover into the normal recipient. In one experiment, donors were injected intravenously with 100 μg of ^{125}I-labeled de-

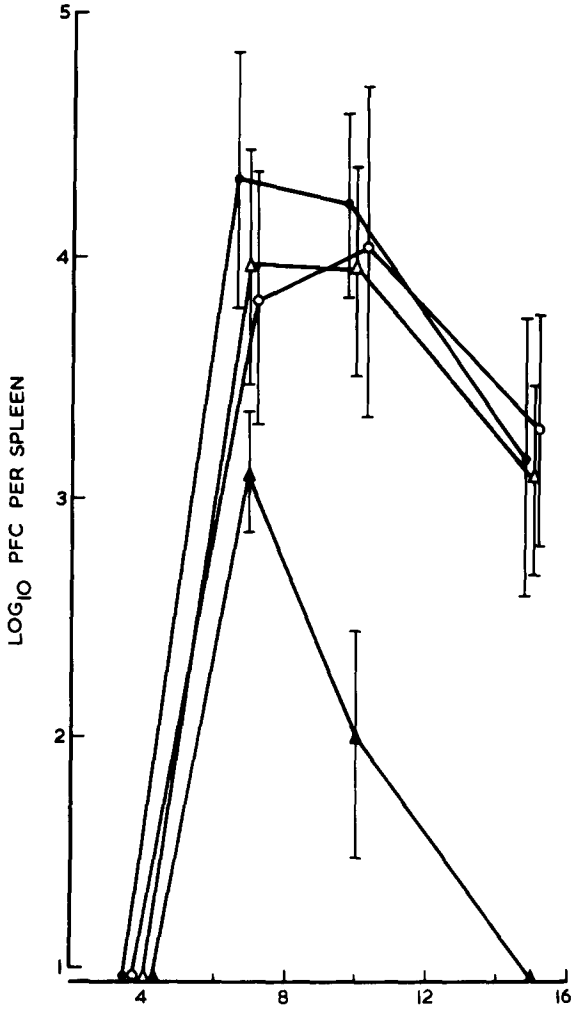

FIG. 2. Time response curve of the inhibitory effect of tolerant spleen cells on the primary adoptive 7S PFC response to FγG in irradiated hosts. 15×10^6 spleen cells from normal mice were given intravenously alone or together with 15×10^6 spleen cells from FγG-tolerant mice. Irradiated recipients were challenged with 500 μg FγG A intraperitoneally and 5×10^8 HRBC intravenously. △, 7S anti-FγG PFC in recipients of normal spleen cells; ▲, 7S anti-FγG PFC in recipients of normal and FγG-tolerant spleen cells; the differences between these two groups are not significant at day 7 ($P > 0.01$) but are significant at day 10 ($P < 0.001$) and 15($P < 0.005$). ○, 7S anti-HRBC PFC in recipients of normal spleen cells; ●, 7S anti-HRBC PFC in recipients of normal and FγG-tolerant spleen cells; the differences between these two groups are not significant. Seven–eight mice per group. The vertical bars represent the 95% confidence limits.

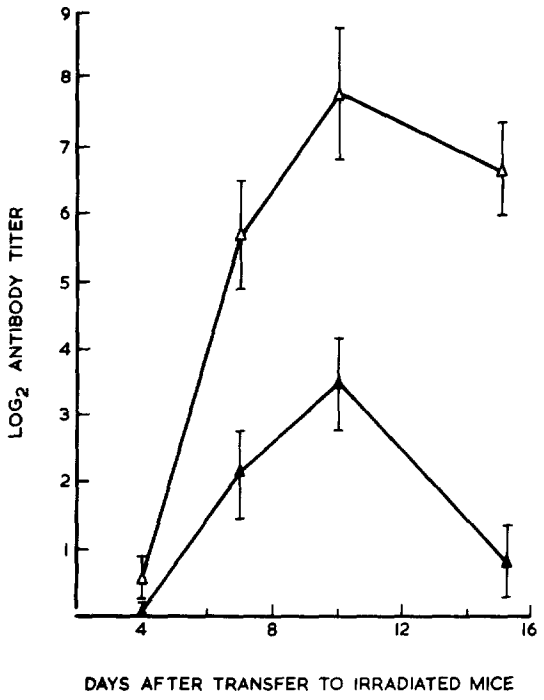

FIG. 3. Time response curve of the inhibitory effect of tolerant spleen cells on the primary adoptive serum antibody response to FγG in irradiated hosts. 15×10^6 spleen cells from normal mice were given intravenously alone or together with 15×10^6 spleen cells from FγG-tolerant mice. Irradiated recipients were challenged with 500 μg FγG A intraperitoneally and 5×10^8 HRBC intravenously. △, anti-FγG serum antibody titer in recipients of normal spleen cells; ▲, anti-FγG serum antibody titer in recipients of normal and FγG-tolerant spleen cells; the differences between these two groups are significant at day 7 ($P < 0.005$), day 10 ($P < 0.01$) and day 15 ($P < 0.01$). Seven–eight mice per group. The vertical bars represent the 95% confidence limits.

aggregated FγG (5×10^5 cpm) at the time of tolerization. The mice were killed by exsanguination and the spleens removed. After thorough washing, an aliquot of 25×10^7 cells was found to contain between 360 and 560 cpm (background 80–90 cpm). In other words, each cell inoculum contained a maximum of approximately 0.02 mg of FγG which, quantitatively speaking, did not constitute a tolerogenic dose in this system.

Effect of Varying Numbers of Tolerant Cells on the Adoptive Secondary Immune Response to FγG.—Further support for the existence of tolerant T cells stems from preliminary results reported previously (27) which suggested that TDL from tolerant donors could in low doses facilitate rather than suppress antibody production by primed (high avidity) B cells. This system was further examined by transferring varying numbers of tolerant spleen cells together with a standard dose of B cells primed to both FγG and the control antigen, DRBC (Table

TABLE IV

Capacity of FγG-Tolerant Spleen Cells to Suppress the Primary Adoptive Response to FγG: Reversal of the θ-Sensitive Suppressor Effect with Purified T Cells

Group	No. and source of cells given*	No. of irradiated recipients‡	PFC/spleen at 7 days§			
			Anti-FγG		Anti-DRBC	
			19S	7S	19S	7S
1	1.5×10^7 normal T cells	8	95(170–55)‖	30(55–25)	70(115–45)	40(70–25)
2	1.5×10^7 normal B cells	8	25(40–15)	50(100–30)	45(75–25)	55(115–25)
3	1.5×10^7 normal T cells + 1.5×10^7 normal B cells	9	250(500–125)	3,040(3,925–2,360)	690(1,115–430)	3,160(4,835–2,065)
4	1.5×10^7 FγG-tolerant spleen cells + 1.5×10^7 normal T cells	7	100(240–40)	20(35–15)	1,305(1,875–905)	3,750(4,725–2,975)
5	1.5×10^7 FγG-tolerant spleen cells treated with anti-θ serum + 1.5×10^7 normal T cells	10	40(60–30)	4,140(5,215–3,285)	950(1,210–750)	3,980(4,935–3,210)
6	1.5×10^7 FγG-tolerant spleen cells + 1.5×10^7 normal B cells	7	200(405–100)	75(160–35)	880(1,165–670)	2,895(5,795–1,450)

P values between groups— 7S PFC to FγG: group 3 cf. 4, <0.001; 4 cf. 5, <0.001; 5 cf. 6, <0.01.

* T cells obtained by column treatment of normal spleen cell suspensions; B cells obtained by treatment of normal spleen cells with anti-θ serum and complement.

‡ Each recipient was given 500 μg FγGA and 5×10^8 DRBC.

§ Data pooled from two experiments.

‖ Geometric mean, upper and lower limits of SE.

TABLE V

Frequency of Antigen-Binding Cells in Normal, Tolerant and Primed Lymphoid Cell Populations

Cell type	Donor	Frequency of small lymphocyte-like cells binding [^{125}I]FγG/10^6 cells*
TDL	Normal	150
	Tolerant	200
	Primed‡	934
Spleen	Normal	300
	Tolerant	535
	Primed‡	800

* Data from one experiment. Comparable results were obtained in another experiment. A minimum of 2×10^4 cells were counted for each cell type.

‡ Primed cells were obtained from donors immunized 6–8 wk previously. Cell suspensions were washed five times before exposure to [^{125}I]FγG.

VII). When 10^7 tolerant cells were used (group 2), a helper effect equivalent to that exerted by the same dose of normal cells (group 5) was observed. By contrast, increasing the dose of tolerant cells to 10^8 resulted in abrogation of antibody production (group 3). The possibility of a nonspecific influence resulting

TABLE VI

*Adoptive Transfer of Tolerance to Normal Syngeneic Recipients**

Group	Treatment of donor mice	No. of spleen cells given	No. of normal recipients‡	PFC/spleen on day 16			
				Anti-FγG		Anti-DRBC	
				19S	7S	19S	7S
1	Tolerization by deaggregated FγG	5×10^7	6	265(540–130)§	6,025(8,570–4,325)	1,470(3,050–710)	1,485(4,615–480)
2	Tolerization by deaggregated FγG	25×10^7	7	185(400–85)	340(890–125)	3,465(4,365–2,740)	2,885(5,335–1,560)
3	No treatment	25×10^7	7	175(385–80)	7,585(9,750–5,900)	2,540(3,485–1,855)	1,780(4,625–685)
4	Tolerization by FγGA and cyclophosphamide‖	25×10^7	7	235(410–135)	4,255(6,140–2,950)	1,155(2,635–510)	3,580(5,945–2,160)

P values between groups—7S PFC to FγG: group 2 cf. 3, <0.05; 3 cf. 4, NS.

* Similar results were obtained in another experiment.

‡ Each recipient was challenged with 500 μg FγGA and pertussis and 5×10^8 DRBC at the time of cell transfer; this was followed by a booster of 100 μg fluid FγG and 5×10^8 DRBC on day 10; PFC were assayed on day 16.

§ Geometric mean, upper and lower limits of SE.

‖ Spleen cells from these mice were unresponsive to FγG on adoptive transfer to irradiated mice but responded well to DRBC.

from administration of such a high dose of cells was excluded by demonstrating (*a*) an enhanced PFC response to the control antigen, DRBC (group 3), and (*b*) a failure of 10^8 normal cells to abrogate the response to FγG at day 7 (group 4).

DISCUSSION

The demonstration of the existence of two classes of lymphocytes with distinctive characteristics has necessitated a reappraisal of the mechanisms involved in regulation of the immune response. Earlier studies have concentrated on defining the facilitating influences of T cells on antibody production (reviewed in 9, 28). More recently, however, evidence, albeit indirect, has been presented by several groups for a suppressive T-cell influence (12–15, 29). The possible existence of a suppressor role for T cells in immune homeostasis is clearly of vital importance particularly to our understanding of self-tolerance and autoimmunity. In this paper direct evidence for "suppressor" T cells was sought by inducing tolerance in adult mice to the "thymus-dependent" antigen FγG and analysing the components of the model in an adoptive transfer system.

Tolerance to FγG was achieved following a single injection of a high dose of deaggregated material. Specificity of the tolerant state was established by demonstrating the concurrent development of a normal response to the noncrossreacting antigens, DRBC or HRBC (Tables I–IV). The unresponsive state was stable on adoptive transfer into irradiated hosts. The fact that no significant amount of labeled tolerogen was detectable on the transferred cells made it unlikely that carryover of antigen maintained tolerance in this situation. This

TABLE VII

*Effect of Cell Dose on the Capacity of FγG-Tolerant Spleen Cells to Collaborate with FγG-Primed B Cells**

Group	No. and source of cells given‡	No. of irradiated recipients§	PFC/spleen at 7 days			
			Anti-FγG		Anti-DRBC	
			19S	7S	19S	7S
1	5×10^6 cells primed to FγG and DRBC	7	40(85–20)‖	120(285–45)	335(515–215)	200(615–65)
2	10^7 tolerant spleen cells + 5×10^6 B cells primed to FγG and DRBC	8	230(380–140)	11,320(13,245–9,675)	575(795–415)	10,750(13,330–8,670)
3	10^8 tolerant spleen cells + 5×10^6 B cells primed to FγG and DRBC	6	85(170–40)	860(2,190–335)	385(915–165)	41,380(45,930–37,280)
4	10^8 normal spleen cells + 5×10^6 B cells primed to FγG and DRBC	6	285(490–120)	56,000(63,250–51,400)	605(1,105–370)	38,750(44,850–33,220)
5	10^7 normal spleen cells + 5×10^6 B cells primed to FγG and DRBC	6	190(345–105)	10,270(14,950–7,955)	520(905–305)	8,990(11,455–7,310)

P values between groups—7S PFC to FγG: group 1 cf. 2, <0.01; 2 cf. 3, <0.05; 3 cf. 4, <0.01; 2 cf. 5, NS. 7S PFC to DRBC: 2 cf. 3, <0.01; 3 cf. 4, NS.

* Another experiment gave similar results.

‡ B cells obtained by treatment of spleen cells primed to FγG and DRBC with anti-θ serum and complement.

§ Each recipient was given 100 μg fluid FγG and 5×10^8 DRBC.

‖ Geometric mean, upper and lower limits of SE.

possibility was rendered even less likely by the finding that the suppressor activity of tolerant spleen cells was abrogated by pretreatment with [^{125}I]FγG or with anti-θ serum (Tables II–IV).

Tolerance could not be abrogated by pretreatment of tolerant cells by trypsin under conditions shown to remove 90% or more of demonstrable surface immunoglobulin. This is difficult to interpret but does, if taken at face value, speak against central suppression of antigen-reactive cells by immune complexes or cross-linkage of receptor sites as envisaged by Diener and Paetkau for B cells (30).

The adoptive primary response of normal spleen cells to FγG was specifically abrogated by addition of tolerant spleen cells. The inhibitory effect was demonstrable both with respect to antibody formation in the spleen (Table II, Fig. 2) and antibody levels in the serum (Fig. 3); the latter data excluded the possibility that antibody was produced by cells in sites other than spleen. Inhibition was shown to be determined by T (θ-bearing) lymphocytes (Table II) which could be inactivated by the suicide technique (31), i.e., by incubating the cells with highly substituted radioactive FγG (Table III).

There was no evidence that tolerance operated at the B-cell level. Thus when tolerant spleen cells were treated with anti-θ serum and adoptively transferred

together with a purified population of normal T cells they gave high responses to FγG (Table IV); these responses were comparable to those obtained with B cells from spleens of normal mice. Similar results have recently been obtained by Ada and Cooper (8) using keyhole limpet hemocyanin in mice and were foreshadowed by the earlier studies of Gershon (13).

Failure to detect a reduction in frequency of antigen-binding cells in the spleen or TDL from tolerant mice (Table V) provided additional confirmation for the nondeletion of B cells. Presumably a similar mechanism may have been operating in tolerance systems previously studied where normal or even increased numbers of antigen-binding cells were reported (reviewed in 8). Moreover, demonstration of cap formation by the receptors specific for FγG on B cells in the tolerant cell population implied that their capacity to recognize antigen, as measured by this criterion, was unimpaired. A comparable phenomenon has been observed by Dunham et al. (32) in their studies on specific B cells in *H-2*-linked unresponsiveness in which the failure of IgG production is also considered to be due to a T-cell lesion (33).

The capacity of tolerant cells to suppress antibody production by normal B cells in irradiated hosts implied that tolerance ought to be transferable to nontolerant recipients. This prediction was indeed verified as shown in Table VI, although large numbers of cells were required. It is of some interest that cell populations rendered unresponsive by the cyclophosphamide technique, which is thought to eliminate specific T cells in this system (26), were unable to suppress antibody production in normal recipients.

Additional evidence in favor of the existence of a suppressor T cell was obtained in experiments designed to test whether tolerant cells could inhibit primed as well as normal B cells. Provided sufficient spleen cells (10^8 per recipient) were given, a highly significant reduction in the adoptive secondary response to FγG was observed (Table VII). By contrast, if low doses (10^7 per recipient) were used, a collaborative response occurred, the magnitude of which was comparable to that obtained with normal spleen cells. In other words, cells from tolerant donors could suppress or help depending on the dose used. The requirement for cell division and differentiation in enabling tolerant cells to engage in both of these activities is now being analysed by pretreatment with a variety of metabolic inhibitors.

These observations raise the question of the mechanism of suppression. There are several possibilities:

(*a*) Tolerance induction could be associated with the formation of immune complexes which might coat antigen-sensitive T cells. T cells with receptors covered by antigen in this form might exert a suppressor rather than a helper effect. Two experiments speak against this: first, the failure of trypsinization to break tolerance in adoptive transfer; second, the demonstration of abrogation of suppression by treatment of tolerant cells with ^{125}I-labeled antigen. In more

critical studies[2] of abrogation of suppressor effects, however, it has been found that the optimal conditions for suicide include overnight incubation of cells with radioactive antigen at 37°C (when complexes could cap off) and the use of approximately five times as much antigen as is required for abrogation of the helper effect. The results of such studies might thus support the immune complex hypothesis.

(*b*) The suppressor effect may represent a T-cell mediated switch in the class or allotype of the antibody produced which is not detected by the enhancing serum used to develop indirect PFC. The marked reduction in serum antibody levels (Fig. 3) tends not to support this suggestion.

(*c*) The capacity of tolerant cells to help at low doses and to inhibit at high doses implied that suppressor activity might be due to "too much help". Thus suprapriming might take place with inhibition of B-cell triggering. There are at least two plausible ways in which this could take place. On the one hand, excess production of nonspecific factors, either by too many T cells or by hyperactive T-cell populations,[3] may limit antigen availability to B cells to such an extent that less antibody is produced. On the other hand, overproduction of a specific T-cell factor (IgT + antigen) may result in saturation of "Fc" receptors on macrophages and direct interaction of high density IgT complexes with immunoglobulin receptors on B cells (34). More detailed dilution analysis, with varying numbers of tolerant cells, is being currently performed in our laboratory to test the validity of these hypotheses. It may be said that, if the suprapriming hypothesis were correct, one might predict that high doses of primed T cells would suppress rather than help and that primed T cells would reinforce the suppressor effect of tolerant T cells. On the other hand, should primed T cells abrogate the suppressor effect of tolerant T cells, it will then be difficult not to accept the possible existence of two distinct types of T cells reacting to similar carrier determinants.

(*d*) An alternative possibility is that a qualitative switch in the T-cell product might have occurred. As a result, a given number of tolerant cells could switch off (or prevent stimulation) of unprimed B cells and yet be still capable of stimulating primed (memory) B cells. In other words, there could be a differential sensitivity of unprimed and primed B cells for the tolerant T-cell product.

(*e*) Two classes of T cells reactive to carrier determinants may exist, one suppressor, the other helper. The recent claim for an enhanced antibody response following passage of normal or primed spleen cells through columns of histamine-rabbit serum albumin-coated sepharose might support such a mechanism (35).

(*f*) The inhibitory effect of tolerant cells on the adoptive FγG response might

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[2] Basten, A. Unpublished observations.

[3] Arrenbrecht, S. and G. F. Mitchell. T-cell dependent helper and suppressive influences in an adoptive IgG antibody response. Manuscript submitted for publication.

represent a delay in differentiation of antibody-forming cell precursors. The demonstration of suppression of PFC and serum antibody levels at 10 and 15 days following cell transfer does, however, render this very unlikely (Figs. 2 and 3).

Although the precise mechanism of suppressor T-cell activity remains in doubt, the experiments illustrated in Tables I, II, and IV do shed some light on the site of action. In all cases a selective reduction in 7S PFC was observed. These results provide further support for a primary role for T cells in regulating the effects of antigen on B cells. By contrast, the 19S PFC, in general, showed no tendency to fall in recipients of tolerant cells. These responses, however, were difficult to interpret since they were usually very low.

The biological significance of a regulatory effect by T cells in the immune response has been extensively discussed (9, 28, 36). Two points are worthy of emphasis here. First, evidence has been obtained that, in at least one form of tolerance, the unresponsive state is determined by a specific suppressive action exerted on B cells by T cells. Such a concept could provide an attractive mechanism for self-surveillance against autoantigens (12). Second, once B cells become primed, for example to a self-antigen, they may, depending on cell ratios, escape the suppressor influence of the corresponding T cells with perpetuation of an autoimmune process.

SUMMARY

Specific immunological tolerance was induced in CBA mice by a single injection of deaggregated fowl immunoglobulin G (FγG). The unresponsive state was stable on adoptive transfer and irreversible by pretreatment of tolerant cells with trypsin. Tolerant spleen cells could suppress the response of normal syngeneic recipients. They also suppressed the adoptive primary response of spleen cells to FγG in irradiated hosts. The inhibitory effect was on the indirect (7S) plaque-forming cell (PFC) response.

Incubation of the tolerant cell population with anti-θ serum and complement reversed the suppressor effect. Furthermore, the addition of purified T cells from normal donors restored the capacity of the anti-θ serum-treated tolerant cells to transfer an adoptive response to FγG. The existence of FγG-reactive B cells was supported by the demonstration of normal numbers of antigen-binding cells in the spleen and thoracic duct lymph from tolerant animals. Moreover, the formation of caps by these cells implied that they could bind antigen normally.

These experiments provided direct evidence for the existence of suppressor T cells in the tolerant population. Further evidence was derived from examination of the effect of antigen "suicide". Tolerant spleen cells were treated with radioactive FγG under conditions known to abrogate T-cell helper function. When these cells were transferred together with normal spleen cells into irradiated hosts, suppression of the primary adoptive response to FγG was no longer ob-

served. Inhibition of an adoptive secondary response to FγG was obtained by transferring tolerant spleen cells with primed B cells provided high doses of tolerant cells were used. By contrast low doses exerted a helper rather than a suppressor effect in this system.

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