

EFFECT OF IDIOTYPE-SPECIFIC SUPPRESSOR T CELLS ON PRIMARY AND SECONDARY RESPONSES*

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When most or all mice of a strain produce antibodies against a particular antigen, some of which share a common idio type, one can suppress the appearance of this idio type by the administration of anti-idiotypic antibodies. Such experiments have been carried out *in vivo* (1-3) and *in vitro* (4). Eichmann (5) demonstrated that the administration of guinea pig IgG2 antibodies caused the appearance of idio type-specific suppressor T cells which could adoptively transfer the suppressed state. To induce such cells he administered a small dose of anti-idiotypic antibody and waited for approximately 8 wk before the transfer. We have found that large numbers of idio type-specific suppressor T cells can be generated by hyperimmunizing the recipient mouse, starting 2 wk after administration of anti-idiotypic antibodies, and allowing a rest period of 8-12 wk after hyperimmunization (6, 7). An average of 6 or 7% of splenic T cells will then form rosettes with autologous erythrocytes¹ (RBC) coated with Fab fragments bearing the idio type (associated with anti-*p*-azophenylarsonate (Ar) antibodies of A/J mice). The rosette-forming lymphocytes include essentially all of the suppressor T cells, and lymphocytes injected as rosettes are at least 10 times as active as suppressors as the original splenic lymphocyte population (7). Most or all of the rosette-forming lymphocytes proved to be T cells, and it was found that the anti-idiotypic receptors are resynthesized by the cells after their removal by tryptic digestion (6). In addition, the RBC have an important role in rosette formation; RBC from heterologous species were much less effective than those from A/J mice and major differences were observed among the RBC of different strains. The capacity of RBC to form rosettes effectively is controlled by one or more genes that are linked to, although not necessarily part of the H-2 locus (8).

Using T-cell populations eluted from nylon wool or rosette-forming T cells as suppressors we have now observed that a secondary idio type response is highly resistant to the effects of suppressor T cells. This was shown by priming recipients before the adoptive transfer of suppressor cells or by cotransferring specifically purified secondary B cells with the suppressor T cells. The experi-

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¹ Abbreviations used in this paper: Ar, *p*-azophenylarsonate; BGG, bovine IgG; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; CRI, cross-reactive idio type; Ed, edestin; Hc, *Limulus polyphemus* hemocyanin; KLH, keyhole limpet hemocyanin; RBC, erythrocytes.

ments do not establish the immediate target of the suppressor T cells.

We have also confirmed a mechanism for suppression based on clonal dominance by B cells (9-11) having receptors specific for the Ar hapten group but lacking the cross-reactive idiotype, and obtained evidence that the two independent mechanisms, involving T and B cells, coexist in a suppressed mouse.

Materials and Methods

Materials. *p*-Aminophenylarsonic acid was obtained from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y., and was recrystallized from a mixture of ethanol and water. Hemocyanin from *Limulus polyphemus* was obtained from Worthington Biochemical Corp. Freehold, N.J., edestin from Sigma Chemical Co., St. Louis, Mo., and bovine IgG (BGG) from Calbiochem, San Diego, Calif. *Limulus* hemocyanin conjugated with Ar was found to give anti-Ar titers and concentrations of cross-reactive idiotype (CRI) very similar to those obtained with keyhole limpet hemocyanin, used in previous studies.

An anti-Thy 1.2 reagent was prepared by immunizing adult male AKR mice with C3H thymocytes. The serum was used without absorption. Rabbit complement with low cytotoxicity was obtained from Dr. Donal Murphy and used at a 1:9 dilution. For one set of experiments the rabbit complement used ("Low Tox") was purchased from Accurate Chemical & Scientific Corp., Co., Hicksville, N.Y. The dilutions of anti-Thy 1.2 and complement used killed less than 10% of splenic lymphocytes when tested separately and 45-55% of normal mouse spleen cells when used in combination. RPMI-1640 medium was obtained from Associated Biomedic. Systems, Inc., Buffalo, N.Y.; fetal calf serum from Grand Island Biological Co., Grand Island, N.Y.; and Hepes buffer from Microbiological Associates, Walkersville, Md.

Methods. The following methods have been described: conjugation of proteins with diazotized *p*-aminophenylarsonic acid (12); preparation of anti-Ar antibodies in the ascitic fluids of A/J mice (13); specific purification of the anti-Ar antibodies, preparation of rabbit anti-idiotypic antibodies against the anti-Ar antibodies of A/J mice, and radioimmunoassays for the idiotype; typically 10 ng of ¹²⁵I-labeled specifically purified anti-Ar antibody from A/J mice was used as ligand (14).

Mice were bled retro-orbitally and their sera assayed for content of anti-Ar antibodies by the method of Klinman et al. (15), which utilizes polyvinyl microtiter plates to adsorb the antigen (BGG-Ar). The method was useful within a range of 2-30 ng of anti-Ar antibodies in the 0.05-ml sample applied to the tray. Proteins were iodinated by the method of Hunter (16), by using carrier-free ¹²⁵I. Fab fragments were prepared from specifically purified A/J anti-Ar antibodies by Porter's method (17) and were purified by passage through DEAE-cellulose in 0.005 M phosphate buffer, pH 8; the Fab fragments are not retained on the column.

A/J mice were suppressed with respect to the CRI associated with anti-Ar antibodies by administering two 0.5-ml portions of a globulin fraction of rabbit anti-idiotypic antibodies (1). The idiotype-binding capacity of the globulin preparation was 60 μg per ml. Suppressed mice were inoculated i.p. five times over a period of 8 wk with 0.5-mg portions of hemocyanin-Ar emulsified in complete Freund's adjuvant (CFA) (volume ratio, 1:1). Lymphocytes to be used for the preparation of idiotype-specific rosettes were obtained from the spleen of such mice, 8-10 wk after the last inoculation.

The method used for the preparation of idiotype-specific rosettes has been described elsewhere (6-8). In most experiments 1×10^7 lymphocytes were mixed with 2×10^8 A/J RBC which had been coated, by the chromium chloride method (18), with 40-100 ng of Fab fragments of specifically purified anti-Ar antibodies (with the CRI) per 10^6 RBC; the fragments were labeled with ¹²⁵I to permit quantitation. Rosettes were separated from free lymphocytes by centrifugation at approximately 100 *g* at room temperature through a Ficoll-Hypaque suspension having a *sp gr* of 1.077. Visual inspection of the pelleted cells indicated a degree of contamination by free lymphocytes of roughly 10%.

Single cell spleen suspensions were prepared by teasing the cells through stainless steel mesh. Their viability, as determined by measurement of trypan blue exclusion, exceeded 80% in each experiment. T cells were purified from single cell suspensions by elution from nylon wool by the method of Julius et al. (19). Typically, 1.5×10^8 splenic lymphocytes in 2 ml of complete medium were applied at 37°C to a 10 cm³ column containing 0.6 g of nylon wool. After incubating for 45

min at 37°C the cells were washed through with 50 ml of ice-cold RPMI-1640 medium containing 10% fetal calf serum free of gamma globulin and collected by centrifugation at 200 *g* for 5 min. Yields ranged from 25 to 36% of the number of lymphocytes applied to the column. In instances when low yields of T cells (<20%) were obtained, we have found that many rosette-forming cells are retained on the column. Cells were adoptively transferred through a tail vein into syngeneic recipients which had received 200 rads of gamma irradiation over a 5-min period, 4 h before the adoptive transfer. An irradiator containing ¹³⁷Cs was used (Sheperd Associates, Glendale, Calif.).

Specific Purification of Lymphocytes with Anti-Ar Receptors. This procedure is similar in principle to that first reported by Wofsy et al. (20), who used hapten-conjugated polyacrylamide beads. Cells were obtained from the spleens of A/J mice that had been hyperimmunized by five inoculations of 0.5-mg portions of hemocyanin-Ar emulsified in CFA (volume ratio of antigen to adjuvant, 1:1). The spleens were removed 4–8 wk after the last injection. The cells were washed three times with 50-ml portions of RPMI-1640 medium containing 10% fetal calf serum and 25 mM glutamine. The medium also contained 0.02 M HEPES buffer, pH 7.0, to increase its buffering capacity. The cells were then passed over a 2-cm column of glass wool in a Pasteur pipette, transferred to a 50-ml polypropylene centrifuge tube with a conical bottom, and mixed with Sephadex G-200 beads, medium grade, to which BGG-Ar had been conjugated by the cyanogen bromide method, as modified by Schlossman and Hudson (21). It was necessary to use an aqueous rather than organic medium during the reaction with cyanogen bromide; otherwise the beads became very sticky. The degree of conjugation was ~0.5 mg of BGG-Ar per cm³ of packed beads. Lower degrees of conjugation resulted in decreased yields of purified cells. The final mixture contained 2 × 10⁸ leukocytes and 5 ml of packed beads in a total of 7 ml. The mixture was allowed to stand at 37°C in an atmosphere containing 7% CO₂ and 93% air and was agitated four or five times over a 30-min period. The tube was then filled with medium, inverted two or three times, and the beads allowed to settle at room temperature for 5 min. The supernate, containing cells, was removed and passed through a Pasteur pipette containing a small amount of cotton wool to remove residual Sephadex beads. The process of mixing with 50-ml portions of medium, settling, and passage through cotton wool was repeated three more times. Very few cells were recovered in the third and fourth tubes.

Lymphocytes were recovered from the beads by exposure to 5 ml of 0.1 M sodium *p*-aminophenylarsonate, pH 8.0. The tube was immersed in a water bath at 37°C and gently agitated for 5 min. It was then filled with medium buffered at pH 7.0. The cells and beads were suspended, the beads were allowed to settle and were separated from the leukocytes by the procedure described above, which was repeated four times. A substantial number of cells was recovered after the first period of settling, a small number was recovered in the second tube, and very few in the third and fourth tubes. The average recovery of lymphocytes after elution with hapten ranged from 0.7 to 2% of the number originally present. The total recovery of cells was 70–80%. Viability in each experiment was approximately 90%.

The cells recovered from the beads by elution with hapten were incubated overnight in complete medium at 37°C in 7% CO₂–93% air and were washed three times with 50-ml portions of the medium to ensure complete removal of the hapten. The viability after standing overnight was at least 80%. Approximately 85% of the recovered leukocytes formed rosettes with A/J RBC coated with bovine serum albumin-Ar. The number of cells killed by treatment with anti-Thy 1.2 antiserum plus complement was not significant.

When the same procedures were carried out with spleen cells from a nonimmune animal, the recovery of leukocytes from the conjugated Sepharose was 0.15% of the number of cells used.

Results

The data in Table I illustrate the effect of priming recipient mice with *Limulus polyphemus* hemocyanin (Hc)-Ar before the adoptive transfer of T cells from suppressed, hyperimmunized syngeneic mice. The recipients were irradiated with 200 rads 4 h before the transfer of T cells, obtained by passage of spleen cells through nylon wool. Each of six recipients which received 1 × 10⁷ T cells produced high titers of anti-Ar antibodies which, however, lacked the CRI. The suppressed state persisted until the end of the experiment (day 70).

TABLE I
Resistance to Suppression by T Cells of CRI in Mice Primed with Antigen*

Group	Mice	Preinjection†	T cells injected	Day 21		Day 70				
				Anti-Ar titer Mean Range	ng Anti-Ar antibody required for 50% inhibition‡	Anti-Ar titer Mean Range	ng Anti-Ar antibody required for 50% inhibition‡			
1	6	None	1×10^7	3.3 (1.7-5.4) <i>mg/ml</i>	>42,000, >82,000, >100,000	>48,000, >85,000, >130,000	0.63 (0.26-1.5) <i>mg/ml</i>	>6,400, >13,000, >38,000	>6,600, >21,000	>9,600, >21,000
2	6	Hc-Ar h - 4	1×10^7	3.9 (2.2-5.9)	3,200, >86,000, >150,000	>40,000, >51,000, >110,000	0.63 (0.47-0.81)	>12,000, >15,000, >20,000	>15,000, >18,000	>15,000, >18,000
3	5	Hc-Ar h - 24	1×10^7	4.4 (1.7-6.7)	270, >160,000	2,200, 3,300, >43,000	0.61 (0.35-0.79)	1,100, >14,000	3,200, >17,000	4,000, >17,000
4	6	Hc-Ar Day - 6	1×10^7	4.2 (2.4-5.0)	8, 9, 10, 10, 12, 430		0.63 (0.44-0.77)	6, 7, 8, 10, 21, 445		
5	3	Hc-Ar h - 24	None	2.6 (1.3-4.6)	48, 60, 130		0.47 (0.38-0.56)	12, 56		
6	3	Hc-Ar Day - 6	None	3.4 (1.6-6.2)	5, 10, 11		0.73 (0.70-0.77)	6, 8, 21		
7	3	None	None	0.41 (0.30-0.51)	30, 31, 88		0.67 (0.54-0.80)	24, 54		

* A single pool of T cells prepared with nylon wool was used. Cells were obtained from 10 suppressed, hyperimmunized mice. Recipients received 200 rads before the adoptive transfer.

† Time of administration of T cells is taken as time zero. A preinjection of 100 μ g of Hc-Ar was given i.p. in CFA at the time specified. After adoptive transfer each mouse received 250 μ g Hc-Ar i.p. in CFA on days 2 and 9 and were bled on day 21. They received a third inoculation after the bleeding and were bled again on day 70.

‡ In the radioimmunoassay for CRI, using 10 ng of 125 I-labeled ligand.

Injection of Hc-Ar 6 days before the adoptive transfer overcame the suppressive effect of the T cells (group 4). Five of six mice in the group produced anti-Ar antibodies with a normal content of CRI and one mouse was partially suppressed. Some effect of preinoculation with Hc-Ar was seen in group 3, which received the antigen 24 h before the adoptive transfer. Three of five mice in the group produced detectable amounts of the CRI, although all the mice were markedly suppressed. Administration of Hc-Ar 4 h before the adoptive transfer had no significant effect. The data obtained with mice in groups 5 and 6 indicated that injection of Hc-Ar alone before irradiation had no effect on the titer of anti-Ar antibodies or CRI which appeared upon subsequent immunization.

The experiments in Table II were similar to those of Table I with the major exception that the T cells inoculated to induce suppression were obtained by preparing rosettes with A/J RBC coated with Fab fragments of specifically purified anti-Ar antibodies bearing the CRI. We have previously reported that the rosette-forming cells are T cells and that they have idio-type-specific suppressor activity (6, 7). The adoptive transfer of 1×10^6 rosettes suppressed the appearance of CRI in each of the eight recipients (group 1). However, the suppression was not as complete as that seen when 1×10^7 T cells were used (Table I). Nevertheless, the degree of suppression was greater than 95% in six mice and was virtually complete in four of the eight mice.

Once again, the injection of Hc-Ar, either 6 days or 4 mo before the adoptive transfer of T cells, prevented suppression of the idio-type. Increasing the dose of T cells to 3×10^6 did not overcome the effect of Hc-Ar administered on day -6; normal concentrations of the idio-type were produced. The higher titers of anti-

TABLE II
Resistance to Suppression of CRI by Idiotypic-Specific Rosettes in Mice Primed with Antigen

Group	Mice	Preinjection*	T cells injected as rosettes	Day 21†		Day 32‡	
				Anti-Ar titer Mean Range	ng Anti-Ar antibody required for 50% inhibition§	Anti-Ar titer Mean Range	ng Anti-Ar antibody required for 50% inhibition
1	8	None	1 × 10 ⁶	0.21 (0.08-0.24)	94, 600, 700, >3,300, >4,500, >4,500, >4,700, >11,000	1.8 (0.55-3.7)	200, 400, 2,800, 3,200, >5,300, >14,000, >31,000, >43,000
2	4	Hc-Ar Day -6	1 × 10 ⁶	0.49 (0.10-1.1)	4, 7, 18, 25	2.6 (0.37-4.8)	25, 63
3	4	Hc-Ar Day -6	3 × 10 ⁶	0.36 (0.09-0.93)	6, 16, 36, 150	1.5 (0.12-3.9)	6, 19, 50
4	4	Hc-Ar Mo -4	1 × 10 ⁶	3.5 (3.4-3.6)	13, 13, 14, 14	5.3 (4.6-5.8)	46, 55, 57
5	4	Hc-Ar Day -6	None	0.28 (0.03-0.63)	8, 16, 22, 81	5.2 (2.5-10.6)	12, 14, 48, 53
6	2	Hc-Ar Mo -4	None	3.1 (2.8-3.3)	11, 13	3.8 (3.6-4.0)	98, 130
7	4	None	None	0.16 (0.04-0.30)	6, 17, 30, 33	0.92 (0.38-1.6)	6, 7, 8, 13

* The time of the adoptive transfer is taken as time zero.

† 100 µg Hc-Ar was given, as the preinjection, i.p. in CFA at the time specified. After adoptive transfer, each mouse received 250 µg Hc-Ar i.p. in CFA on days 2, 9, and 21. They were bled before the inoculation on day 21 and then again on day 32.

§ See footnote §, Table I.

Ar antibody in mice that received the antigen 4 mo before the adoptive transfer was probably due to the presence of memory cells. The serum of each of these mice was tested by radioimmunoassay just before the adoptive transfer and found not to contain significant concentrations of anti-Ar antibodies.

The data in Table III are related, first, to the question of whether the resistance to suppression of idiotypic after priming with Hc-Ar (Tables I and II) is attributable to the priming of carrier-specific helper cells. The results obtained with the mice of group 2, Table III, indicate that this is not the case. Priming with unconjugated hemocyanin 6 days before the adoptive transfer did not affect the capacity of suppressor T cells to prevent the appearance of the CRI. Further evidence that the hapten group was responsible for resistance to suppression was obtained by injecting another conjugate, edestin-Ar, 6 days before the adoptive transfer of suppressor T cells (group 3). All of the mice in the group produced significant amounts of the CRI upon subsequent immunization with Hc-Ar.

The results obtained with the mice of groups 4 and 5 indicate that the suppressor T cells do not act exclusively on cells that recognize the carrier. Suppression was effective when the mice were subsequently challenged with edestin-Ar or BGG-Ar as well as hemocyanin-Ar (group 1), despite the fact that the suppressor cells were generated in mice treated with anti-idiotypic and then with Hc-Ar.

Data for groups 6-8 indicate that each of the conjugates used could elicit anti-Ar antibodies possessing the CRI in nonsuppressed mice, although the average concentration, per unit weight of anti-Ar antibodies, was somewhat lower in the group that received edestin-Ar. An interesting result was noted when the mice were immunized with BGG-Ar (group 8). The antibodies obtained on day 17 had a relatively low concentration of the idiotypic as compared with those

TABLE III
*Effect of Prepriming Recipients with Hemocyanin or Edestin (Ed)-Ar, or Subsequent Challenge with the Ar Group on Different Carriers, on Suppression of CRI by T Cells**

Group	Mice	Preinjection†	T cells injected	Antigen challenge	Day 17		Day 49	
					Anti-Ar titer Mean Range	ng Anti-Ar antibody required for 50% inhibition§	Anti-Ar titer Mean Range	ng Anti-Ar antibody required for 50% inhibition
1	6	None	1 × 10 ⁷	Hc-Ar	0.36 (0.11-0.49)	>2,600, >8,800, >9,000, >10,000, >11,000, >12,000	2.0 (1.4-2.8)	>27,000, >38,000, >49,000, >54,000, >56,000, >69,000
2	6	Hc Day -6	1 × 10 ⁷	Hc-Ar	0.28 (0.09-0.62)	>2,300, >3,300, >6,400, >7,000, >8,000, >16,000	1.7 (0.11-4.5)	>15,000, >29,000, >31,000, >37,000, >39,000, >113,000
3	9	Ed-Ar Day -6	1 × 10 ⁷	Hc-Ar	1.0 (0.04-1.9)	13, 40, 58, 66, 69, 150, 200, 400, 600	1.2 (0.42-1.8)	60, 140, 350, 400, 900, 980, 1,500, 1,400
4	6	None	1 × 10 ⁷	Ed-Ar	0.21 (0.06-0.52)	1,100, >1,600, >2,800, >4,000, >5,000, >13,000	3.7 (0.51-7.4)	>3,300, >5,100, >6,700, >65,000, >77,000
5	6	None	1 × 10 ⁷	BGG-Ar	0.35 (0.21-0.65)	>5,200, >6,700, >7,300, >7,500, >10,000, >16,000	2.9 (0.9-5.6)	>22,000, >45,000, >49,000, >57,000, >132,000, >139,000
6	5	None	None	Hc-Ar	0.033 (0.023-0.060)	11, 12, 29, 170, 300	3.6 (2.8-3.8)	10, 11, 36, 38, 40
7	6	None	None	Ed-Ar	0.023 (0.016-0.039)	18, 27, 200, 200, 400, 400	7.9 (4.3-10.7)	10, 32, 130, 970, 1,700
8	5	None	None	BGG-Ar	0.074 (0.030-0.094)	62, 200, 190, 430, 870	2.2 (1.3-3.8)	11, 20, 31, 40, 61

* See footnote *, Table I.

† See footnote †, Table I. After adoptive transfer, each mouse received 250 μg Hc-Ar i.p. in CFA on days 2, 9, and 21.

§ See footnote §, Table I.

antibodies present at day 49. Some evidence of the same trend was noted when Hc-Ar was the immunogen (group 6). Further experiments are needed to determine whether this occurs with regularity.

The data thus indicate that priming with protein-Ar conjugates before the adoptive transfer of suppressor T cells prevents the suppression of idotype that is otherwise observed on subsequent immunization with a protein-Ar conjugate. One possible explanation is that the secondary B cells generated on priming are resistant to suppression, either because of the relatively large number of cells present or because of their intrinsic characteristics. To obtain more direct evidence on this question, cells with anti-Ar receptors were specifically purified by using Sephadex to which BGG-Ar was bound and eluting the cells with hapten as described under Methods.

Experiments with Specifically Purified Cells Having Anti-Ar Receptors. For use in the experiments to be described next, cells with anti-Ar receptors were specifically purified from the spleens of hyperimmunized A/J mice, both suppressed and nonsuppressed with respect to the CRI. Treatment with anti-Thy 1.2 and complement failed to kill a significant proportion of the cells whereas treatment of normal splenic lymphocytes with the same reagents killed 45-55% of the lymphocytes. To test for the presence of anti-Ar receptors, rosettes were prepared by using A/J RBC to which BSA-Ar was conjugated by the chromium chloride method. The recovery of splenic lymphocytes from the Sephadex was 0.75% of the total spleen cell population. (In other experiments the recoveries have been as high as 2%). Before rosetting, the cells were allowed to stand in culture medium overnight at 37°C. The percentages of cells that

TABLE IV
Resistance to Suppression Conferred by Specifically-Purified Secondary B Cells Adoptively Transferred with Suppressor T Cells*

Group	Mice	Hyperimmune Ar-specific B cells injected*	T-cell rosettes injected†	Day 17		Day 27	
				Anti-Ar titer§ Mean Range	ng Anti-Ar antibody required for 50% inhibition	Anti-Ar titer Mean (Range)	ng Anti-Ar antibody required for 50% inhibition
1	4	None	1 × 10 ⁶	0.38 (0.02-0.72)	1,900, >7,500, >14,000, >18,000	1.7 (0.98-3.5)	7,700, 9,800, >64,000, / >139,000
2	3	2 × 10 ⁵	1 × 10 ⁶	0.30 (0.09-0.45)	57, 400, 450	1.7 (0.61-3.4)	34, 110, 600
3	3	1 × 10 ⁵	1 × 10 ⁶	0.23 (0.09-0.32)	1,600, 2,000, >7,300	0.62 (0.25-1.3)	130, 320, 2,500
4	3	0.5 × 10 ⁵	1 × 10 ⁶	0.32 (0.28-0.38)	>7,000, >7,500, >9,500	0.30 (0.28-0.34)	34, 75, 2,800
5	4	2 × 10 ⁵	None	0.12 (0.04-0.22)	27, 35, 40, 50	0.94 (0.03-1.9)	85, 180
6	4	None	None	0.13 (0.03-0.25)	24, 30, 44, 64	3.3 (0.98-6.4)	18, 38, 64, 98

* B Cells with anti-Ar receptors were specifically purified from the spleens of four hyperimmunized A/J mice, and were then pooled.

† Lymphocytes used for preparing the rosettes used for suppression were from the spleens of three suppressed, hyperimmunized mice (6, 7). The B cells and the rosettes were mixed and incubated at 37°C for 1 h before i.v. adoptive transfer into A/J recipients that had received 200 rads.

§ Each recipient was given 250 µg of Hc-Ar i.p. in CFA on days 2, 10, and 21 after the adoptive transfer.

formed rosettes were 90 and 84% for two preparations of cells from nonsuppressed mice (Tables IV and V) and 86% for the cells of idiotypically suppressed mice (Table V). From 2 to 3% of the spleen cells from hyperimmunized animals formed hapten-specific rosettes. Spleen cells from nonimmunized animals did not form a significant number of rosettes in each of three experiments carried out. From their properties, it would appear that the specifically purified cells consist largely or almost entirely of B cells.

To test the effect of the purified cells on suppression, they were mixed in varying proportions with idio-type-specific rosettes prepared with lymphocytes from a pool of spleens of three suppressed hyperimmunized A/J mice. The mixture of the two cell types was incubated in a vol of 2 ml at 37°C for 1 h, then injected intravenously into recipient mice that had received 200 rads 4 h before the transfer. The mice were then immunized with hemocyanin-Ar according to the schedule given in a footnote of Table IV. Administration of 1 × 10⁶ T cell rosettes alone completely suppressed the appearance of the CRI in the subsequently immunized recipients (group 1, Table IV). In contrast, when the rosettes were mixed with 2 × 10⁵ purified B cells, the animals produced significant amounts of the CRI. When 1 × 10⁵ or 0.5 × 10⁵ purified cells were mixed with the rosettes, the amount of CRI present on day 17 was very low, but it increased substantially in two of three mice in each group by day 27; i.e., the suppression appeared to be transient in four of the six mice.

The experiments were repeated in an experiment (not shown) by using 2 × 10⁵ specifically purified cells with anti-Ar receptors that had been treated with anti-Thy 1.2 antiserum plus complement and 1 × 10⁷ suppressor T cells (eluted from nylon wool). Four recipient mice were used of which three survived the experiment. Each mouse possessed a high titer of CRI on day 17; 50-65 ng of anti-Ar antibody was required for 50% inhibition. Five control mice which received only the T cells were completely suppressed with respect to the CRI. Thus, administration of secondary B cells with anti-Ar specificity greatly increases the resistance to suppression by idio-type-specific suppressor T cells.

The experiments of Table V were carried out to determine whether B cells

TABLE V
Adoptive Transfer of Suppression of Idiotype by Specifically Purified B Cells from Suppressed, Hyperimmunized A/J Mice

Group	Mice	Cell preparation transferred	Treatment of cells	Cells transferred	Anti-Ar titer [§] Mean Range	ng Anti-Ar antibody required for 50% inhibition
					mg/ml	
1	4	Spec. purified suppressed cells*	NMS† + C	2 × 10 ⁵	2.3 (1.3-3.7)	64, 13,000, >34,000, >94,000
2	4		NMS + C	1 × 10 ⁵	1.9 (0.84-2.7)	10, >24,000, 27,000, >50,000
3	4		NMS + C	0.5 × 10 ⁵	1.8 (1.1-2.9)	140, 400, 1,900, 2,900
4	4		Anti-Thy 1.2 + C	2 × 10 ⁵	1.2 (0.46-2.4)	1,000, 4,000, 9,000, >59,000
5	4		Anti-Thy 1.2 + C	1 × 10 ⁵	1.6 (0.88-2.0)	61, 18,000, >40,000
6	4		Anti-Thy 1.2 + C	0.5 × 10 ⁵	0.33 (0.34-1.4)	8, 17, 550, 2,000
7	4	Nonsuppressed cells*	NMS + C	2 × 10 ⁵	0.92 (0.26-1.4)	43, 79, 83, 86
8	4		Anti-Thy 1.2 + C	2 × 10 ⁵	2.5 (1.1-3.8)	25, 34, 120, 150
9	4	None	None	None	2.1 (0.89-2.9)	28, 46, 67, 110
10	4	T-cell rosettes	None	1 × 10 ⁵	1.4 (0.18-2.9)	37, 9,000, >24,000, >40,000

* B cells with anti-Ar specificity were specifically purified from the spleens of hyperimmunized, suppressed, or nonsuppressed A/J mice.

† Normal mouse serum.

§ Subsequent to the adoptive transfer each mouse received 250 µg of Hc-Ar i.p. in CFA on days 2 and 9 and was bled on day 21.

specifically purified from a hyperimmune suppressed animal, and treated with anti-Thy 1.2 antiserum and complement, are capable of adoptively transferring the idiotypically suppressed state into mildly irradiated recipients. We have reported elsewhere (9-11) on the adoptive transfer of suppression by B cells and attributed it to clonal dominance of large numbers of memory cells with anti-Ar receptors lacking the CRI. In the present experiments it was possible to use smaller numbers of cells since they had been specifically purified; after treatment with anti-Thy 1.2 and complement the number of contaminating T cells must have been very low. The data in Table V indicate that 2 × 10⁵ or 1 × 10⁵ purified B cells from suppressed mice were capable of adoptively transferring the suppressed state. 12 of the 15 mice in groups 1, 2, 4, and 5 produced anti-Ar antibodies which lacked detectable CRI. The transfer of 0.5 × 10⁵ B cells (groups 3 and 6) was somewhat less effective in inducing the suppressed state. Treatment with anti-Thy 1.2 and complement had no significant effect on the suppressive activity of the purified B cells (groups 4-6).

Controls were run in which the specifically purified B cells with anti-Ar receptors were obtained from nonsuppressed mice. The transfer of such cells had no significant effect either on anti-Ar antibody titers or the concentration of CRI (groups 7 and 8).

Comparison of these results with those obtained with mice of group 9, which were irradiated but did not receive cells, indicate that the adoptive transfer of B cells from either suppressed or nonsuppressed mice did not significantly affect anti-Ar antibody titers on day 21.

The mice of group 10 received rosettes made with T cells from the same group of suppressed, hyperimmunized mice that had provided the B cells used in the experiments of groups 1-6. As expected from earlier data, the adoptive transfer of rosettes suppressed the appearance of the CRI on subsequent immunization with Hc-Ar. This indicates that a single pool of spleen cells contains both T cells and B cells that are independently capable of adoptively transferring the suppressed state.

Discussion

These experiments demonstrate that a secondary response is far more resistant than the primary response to the action of idiotypic-specific suppressor T cells. A/J mice which had been primed with antigen (Hc-Ar) 6 days before mild irradiation and the adoptive transfer of suppressor T cells, produced normal concentrations of the CRI upon subsequent immunization with KLH-Ar. A small but significant effect was also noted when the mice were primed 24 h (but not 4 h) before the transfer. The resistance to suppression was long-lived; similar results were observed after periods of 21 or 70 days (the last inoculation was on day 27). The state of suppression persisted for the duration of the experiment in those mice which had not been primed. On day 70 the titers of anti-Ar antibody were still moderately high. We have never observed escape from the suppressed state once immunization is initiated, although there is a gradual recovery if antigen is withheld (22).

Similar results, with respect to the effect of priming recipients before the adoptive transfer, were obtained when the T cells were administered either in the form of a nylon wool eluate (Table I) or as rosettes, with the lymphocytes bound to A/J RBC coated with Fab fragments bearing the CRI (Table II). The use of a smaller number of T cells (1×10^6) in the form of rosettes was based on our previous observation that less than one-tenth as many rosettes, as compared to unfractionated T cells, are required to induce the suppressed state. (Previous experiments have shown that the rosette-forming cells are largely or entirely T cells [6, 7]). With the rosettes, an additional experiment was carried out in which the recipients were primed with a single injection of Hc-Ar 4 mo before the adoptive transfer. The mice in this group produced normal amounts of the CRI upon subsequent immunization; i.e., they were not suppressed. Just before the adoptive transfer these mice were bled and anti-Ar antibodies were not detected (concentration, $<2 \mu\text{g/ml}$). The experiment therefore indicates that the cells induced by the injection of Hc-Ar either have a long half-life or were replicated during the 4-mo period. As suggested below the relevant cells appear to be B cells.

The results in Table III indicate that the resistant cell, induced upon priming with Hc-Ar, is not a carrier-specific T cell. Animals primed with Hc, rather than Hc-Ar, were not resistant to suppression, whereas mice primed with edestin-Ar produced significant amounts of the CRI.

The results obtained with groups 4 and 5, Table III, indicate that suppressor T cells are effective irrespective of the carrier used for subsequent immunization. Mice challenged with edestin-Ar or BGG-Ar after receiving suppressor T cells did not produce significant amounts of the CRI, despite the fact that the T cells were from donors immunized with Hc-Ar after idiotypic suppression. This provides evidence that the target of the suppressor cell is not a cell with exclusive specificity for carrier.

More direct evidence that resistance is attributable to the presence of secondary B cells was obtained through the use of specifically purified cells with anti-Ar receptors from hyperimmune mice. When 2×10^5 cells that were eluted from a column of Sephadex-BGG-Ar were treated with anti-Thy 1.2 and complement, then mixed and cotransferred with the suppressor T cells, the

recipients produced significant amounts of the CRI.

When smaller numbers of Ar-specific B cells (5×10^4 or 1×10^5) were tested each of the six recipient mice had a considerably higher content of the CRI, per unit weight of anti-Ar antibody, on day 27 as compared to day 17. Perhaps the simplest explanation is that the antibodies present on day 17 were largely produced by descendants of primary B cells in the recipient animal, which were subject to idiotypic suppression, but that by day 27 the small number of secondary B cells that were transferred had multiplied so that they dominated the immune response and terminated the suppressed state.

The observation that the spleen cells from suppressed, hyperimmunized mice contain both B cells and T cells that can independently transfer the suppressed state adoptively suggests that, *in vivo*, these mechanisms are complementary and provide a powerful means of maintaining a state of suppression once immunization has been initiated. As indicated earlier, we have never observed recovery from the suppressed state once immunization has been initiated.

Although the results clearly indicate that secondary B cells are resistant to suppression by idiotype-specific suppressor T cells, they do not establish the primary target of the suppressors. Since, as shown by rosetting (6, 7), the suppressor cells can bind to Fab fragments bearing the idiotype, they appear to have receptors with anti-idiotypic specificity. One would then predict that the target is either a T cell or a B cell with receptors carrying the CRI. K. Eichmann (personal communication) has evidence for the existence of two classes of helper T cells in strain A mice, each capable of interacting with group A streptococci. Type 1 carries the CRI characteristic of anti-group A streptococcal antibodies in the A strain. Type 2 lacks the idiotype. Type 1 T cells can stimulate the proliferation of T helper cells with anti-idiotypic receptors, which may act by stimulating B cells carrying receptors with the CRI. A possible interpretation of the present results is that idiotypic suppression inactivated T helper cells bearing the idiotype² but not those lacking the CRI; and that a primary response requires the presence of both types of helper cell whereas the type 2 cell (unaffected by antiidiotype) suffices to trigger a secondary response. The existence of two types of helper T cells, one of which has specificity for immunoglobulin receptors, has also been proposed by Janeway et al. (23), and by Tada et al. (24). The possibility that an idiotype-bearing B cell is the target must also be considered.

Ward et al. (25) demonstrated a reduction in concentration of the anti-Ar idiotype when carrier-primed helper cells were adoptively transferred from idiotypically suppressed (as compared to nonsuppressed) donors; secondary B cells primed to the Ar group were transferred at the same time and the irradiated recipients were immunized with keyhole limpet hemocyanin (KLH)-Ar. Their results differ somewhat from ours in that they observed partial

² A distinction between our system and Eichmann's is that in the case of the streptococcal antigen the antigen-specific helper cell can carry the CRI, whereas a helper cell specific for hemocyanin would probably not have receptors with the anti-Ar CRI. In our system the helper cell with idiotypic receptors could, in this model, be stimulated by hapten and in turn generate anti-idiotypic helpers, but might not itself play a direct role (unless some help is mediated *via* hapten groups on the immunogen).

suppression of the secondary idiotype response. The difference may reside in the numbers of carrier-specific helper cells present in the two sets of experiments.

Resistance of secondary cells to suppression has been reported by Pierce and Klinman (26), who challenged mice with a hapten-protein conjugate, lethally irradiated them, and then adoptively transferred syngeneic cells as a source of B cells. This prior challenge of the recipients substantially reduced the number of hapten-responsive cells in a transferred population of primary, but not secondary B cells. Since the ability to suppress the response of the B cells was allotype-linked, they postulated that the observed suppression may be at the level of idiotype. The resistance of secondary cells to such suppression would be concordant with the present data.

In an earlier report we showed that the cross-reactive idiotype can be suppressed by anti-idiotypic antibodies (in contrast to suppressor T cells) in animals primed by a single injection of KLH-Ar (27). This may reflect a different mechanism of action of the two suppressive agents. One possibility is that a high concentration of antiidiotypic antibodies can directly inactivate primed B cells, at least for a limited period of time.

Summary

Previous reports have shown that suppression of idiotype can be adoptively transferred by T cells, or by rosettes containing T cells with anti-idiotypic receptors, from an idiotypically suppressed, syngeneic mouse. The present data indicate that secondary B cells are highly resistant to such suppression. Priming recipients to the relevant hapten, *p*-azophenylarsonate, 6 days or 4 mo before the adoptive transfer prevented suppression. This was independent of the carrier used for the hapten group during priming or subsequent immunization, suggesting that resistance to suppression is attributable to secondary cells with specificity for the hapten. The effect of suppressor T cells could also be overcome by mixing them with specifically purified B cells having receptors for the hapten group before the adoptive transfer. Adoptive transfer of the suppressed state by specifically purified B cells from suppressed, hyperimmunized animals confirmed our previous finding that the suppression of idiotype can also be caused by B cells lacking idiotypic receptors, evidently through a mechanism involving clonal dominance. Possible mechanisms of idiotypic suppression by T cells are discussed.

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