PRESENCE ON IDIOTYPE-SPECIFIC SUPPRESSOR T CELLS OF RECEPTORS THAT INTERACT WITH MOLECULES BEARING THE IDIOTYPE*

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When immunized with keyhole limpet hemocyanin-*p*-azophenylarsonate (KLH-Ar),[1] all A/J mice produce anti-Ar antibodies of which a substantial proportion share a cross-reactive idiotype (CRI) (1). If, however, the mice are treated with rabbit anti-idiotypic (anti-id) antiserum before immunization, they produce anti-Ar antibodies lacking the CRI, although there is little or no effect on the total concentration of anti-Ar antibodies that are present (2, 3). Eichmann has shown that T cells from an idiotypically suppressed mouse can be used to transfer adoptively the suppressed state into a mildly irradiated syngeneic recipient (4). We have confirmed his results and have also obtained data indicating that B cells from an animal that is suppressed and then hyperimmunized are capable of transferring suppression, presumably through direct competition of a large number of B cells bearing nonidiotypic receptors with a relatively small number of B cells possessing receptors with the CRI in a nonimmunized animal (reference 5 and K. A. Ward, H. Cantor, and A. Nisonoff, unpublished data).

More recently, we have shown that T cells obtained from the spleen of a suppressed, hyperimmunized mouse form rosettes with A/J red blood cells (RBC) coated with Fab fragments of anti-Ar antibodies possessing the CRI (6, 7).[2] The responsible receptors on the T cells are synthesized endogenously and not passively adsorbed, as shown by experiments in which the receptors were removed by treatment with trypsin and reappeared upon standing in tissue culture (7).[2] In order to obtain an optimal number of idiotype-specific rosette-forming T cells, it is necessary to allow a rest period of 6–12 wk after immunization of the suppressed mice. At this time, the percentage of T cells that form rosettes generally varies between 6 and 10% of the total T-cell population. Specificity for the idiotype has been demonstrated by a variety of control experiments (6, 7).[2]

Since T cells from suppressed animals are capable of adoptively transferring suppression, it seemed possible that the rosette-forming population might include the suppressor T cells. The data to be presented here indicate that this is the case; the suppressor T-cell population can be depleted by removing those T cells which form idiotype-specific rosettes. Furthermore, the rosetted cells are

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[1] *Abbreviations used in this paper:* anti-id, anti-idiotypic; Ar, *p*-azophenylarsonate; CFA, complete Freund's adjuvant; CRI, cross-reactive idiotype; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline, pH 7.0; RFC, rosette-forming cells; T(Ig^-), T cells prepared by the method of Parish et al. (Materials and Methods).

[2] Owen, F. L., S-T. Ju, and A. Nisonoff. 1977. Binding to idiotypic determinants of large population of T cells in idiotypically suppressed mice. *Proc. Natl. Acad. Sci. U. S. A.* In press.

active as suppressors. The results do not establish whether all of the rosette-forming cells are suppressor cells.

Materials and Methods

Materials. Adult A/J male mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, or were first generation descendants of mice from that source. Complete Freund's adjuvant (CFA) was obtained from the Difco Laboratories, Detroit, Mich.; KLH from Calbiochem, La Jolla, Calif.; and *p*-aminophenylarsonic acid from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y. The latter compound was recrystallized from a mixture of water and ethanol. Ficoll was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. and Hypaque from Winthrop Laboratories, New York. Anti-Thy-1.2 antiserum was prepared by the method of Reif and Allen (8). The source of rabbit complement (C) and the method used for absorption are described elsewhere.[2] The activity and cytotoxicity are indicated by the data presented under Results. T cells were prepared from spleen by the method of Julius et al. (9), using nylon wool, or by a minor modification of the method of Parish et al. (10), in which spleen cells are coated with an IgG fraction of rabbit anti-mouse Fab and adsorbed to A/J RBC coated, using $CrCl_3$, with an IgG fraction of goat anti-rabbit Fab. Our modification consisted in the use of Fab fragments of rabbit anti-mouse Fab, rather than an IgG fraction, and of A/J RBC instead of sheep RBC. The B lymphocytes are adsorbed to the coated RBC while T cells, null cells, and glass-adherent cells remain free. The complexes of B cells and RBC are sedimented by centrifugation, which also removes free RBC. Purity of the T-cell preparations, estimated by treatment with anti-Thy-1.2 and C, is discussed under Results. The B cells were not used. Macrophages were not observed in rosettes.

Preparation of Anti-id Antibodies and Assays for CRI. We have previously described the following procedures: preparation of KLH-Ar; specific purification of anti-Ar antibodies; preparation of rabbit anti-id antisera against anti-Ar antibodies of the A/J strain and proof of idiotypic specificity; radioimmunoassay for the CRI in unlabeled samples (1). The assay is based on a double antibody procedure, using 10 ng of ^{125}I-labeled specifically purified anti-Ar antibody as ligand, rabbit anti-id antiserum, and goat anti-rabbit Fc to precipitate the complexes; 0.25 mg of mouse IgG is also present in each test mixture. The presence of the idiotype in an unknown, unlabeled sample is determined through its capacity to inhibit the binding of the labeled ligand. Tests for idiotypic specificity of absorbed rabbit anti-id antiserum were carried out as described previously (1, 2). Total concentrations of anti-Ar antibodies were determined by a minor modification of the method of Klinman et al. (11), c.f., Ju et al. (12), in which the mouse antiserum is exposed to a polyvinyl surface coated with bovine serum albumin-Ar. This is followed by the addition of ^{125}I-labeled specifically purified rabbit anti-mouse IgG; the uptake of label provides a measure of the anti-Ar antibody content of the mouse serum. The method is standardized with sera containing known concentrations of anti-Ar antibodies, as determined by precipitin analysis. The useful range is 2–40 ng of anti-Ar antibody in 0.1 ml. All assays were carried out in duplicate.

Immunological Suppression of CRI. This was accomplished by injecting two 0.5-ml portions of rabbit anti-id antiserum (total idiotype-binding capacity 60–80 μg) into male A/J mice, 6 wk of age. The injections were given 3 days apart. Starting 2 wk later, the mice were hyperimmunized with four injections of 0.5-mg portions of KLH-Ar in CFA over a period of 5 wk. The volume ratio of adjuvant to antigen was 1:1. These mice did not produce detectable quantities of the CRI but had serum titers of anti-Ar antibody between 1.0 and 6.4 mg/ml (mean, 3.9 mg/ml). The mice were allowed to rest for 8 wk before sacrifice and preparation of rosettes. The requirement for a rest period has been shown previously (6, 7).

Other Methods. Proteins were labeled with ^{125}I by using chloramine-T (13). Fab fragments were prepared from nonspecific IgG or specifically purified anti-Ar antibodies by digestion with papain (14). They were purified by passage through DEAE-cellulose in 0.005 M phosphate buffer, pH 8.0; the Fab fragments are not retained on the column. Purity of the fragments was ascertained by Ouchterlony analysis. Fab fragments were coupled to A/J RBC with $CrCl_3$ by a modification of the method of Jandl and Simmons (15), as described elsewhere (6).[2] The Fab fragments were lightly iodinated with ^{125}I to permit quantitation of the amount coupled to the RBC; values ranged from 30 to 50 ng/10^6 RBC. Rosettes were prepared by mixing 10^7 lymphocytes with a 20-fold excess of RBC at room temperature in 0.2 ml of RPMI-1640 medium containing 5 μg/ml gentamycin, 3 ×

10^{-6} M glutamine, streptomycin, and penicillin. The mixture was centrifuged at 500 *g* for 5 min, then allowed to stand for 30 min at room temperature and 30 min in an ice bath. The cells were resuspended in the supernate and layered over a Ficoll-Hypaque solution (specific gravity, 1.077). The mixture was centrifuged for 15 min at 450 *g*. Free lymphocytes remained at the interface while rosettes and RBC were sedimented. Before this sedimentation, the percentage of rosette-forming cells (RFC) was determined on a 0.1-ml portion of the mixture by adding 0.1 ml of a solution containing 1% glutaraldehyde and 5% crystal violet in phosphate-buffered saline, pH 7.0 (PBS) (10). The dye selectively stains the lymphocytes. The rosettes were counted at a magnification of 630, using a Zeiss microscope fitted with a projection screen (Carl Zeiss, Inc., New York).

Results

The data presented in Tables I, II, and III were obtained by using splenic lymphocytes from 13 mice that had been suppressed with respect to the CRI, hyperimmunized with KLH-Ar, and allowed to rest for 9 wk before sacrifice. Lymphocytes were prepared from the 13 spleens and pooled.

The data in Table I show that 8.0% of the splenic lymphocytes, 5.0% of T cells prepared by passage through nylon wool, and 10.3% of T cells prepared by the method of Parish et al. (10) formed rosettes with A/J RBC coated with the Fab fragments of A/J anti-Ar antibodies possessing the CRI. No rosettes were detected when the lymphocytes were mixed with RBC coated with Fab fragments prepared from nonspecific A/J IgG.

That the receptors on the lymphocytes were reacting with idiotypic determinants is shown by the data on inhibition in Table I. 1 μl of serum containing 3.5 μg of anti-Ar antibody bearing the CRI inhibited rosette formation almost completely, whereas 5 μl of normal A/J serum, or 5 μl of A/J serum containing 18.5 μg of anti-Ar antibodies lacking the CRI, had a minimal effect on rosetting. The large percentages of lymphocytes forming idiotype-specific rosettes confirm previous observations with other suppressed mice (6, 7).

The data in Table II indicate that most of the rosette-forming lymphocytes are T cells. The results in the first three columns show the effectiveness of the anti-Thy-1.2 reagent, while the results in the last column of the table indicate that most of the rosette-forming lymphocytes are killed by anti-Thy-1.2 plus C. To obtain these lymphocytes, the rosettes were first separated from free lymphocytes by centrifuging through a Ficoll-Hypaque solution. The RBC were lysed when warmed to 37°C in the presence of the rabbit serum that was used as a source of C. This is attributable to the fragility of RBC treated with $CrCl_3$. The lymphocytes that had formed rosettes were not killed by the anti-Thy-1.2 reagent and only a small percentage were killed by exposure to C, whereas a mixture of the two reagents killed 92% of the cells. The number of cells killed was estimated by counting the number of lymphocytes remaining intact after each treatment, and which retained the ability to exclude trypan blue.

Table III presents data obtained after adoptive transfers of the T cells, prepared by the method of Parish et al. (10), from the pool of 13 spleens already described. Adoptive transfers were carried out with T cells, T cells depleted of idiotype-specific rosette-forming lymphocytes, and the rosettes themselves. Recipients were irradiated (200 R) adult A/J mice. Immunization consisted of four inoculations of 0.25 mg of KLH-Ar in CFA on days 3, 17, 24, and 31 after the adoptive transfer; a 1:1 volume-ratio of adjuvant to antigen solution was used. The immunized recipients were bled on day 35. In the control groups, which

TABLE I

*Rosette-Forming Splenic T Lymphocytes in Suppressed, Hyperimmunized A/J Mice**

Red cell coat	Inhibitor	Source of lymphocytes		
		Spleen	T (nylon wool)	T(Ig^-)‡
		% RFC		
Fab id§	None	8.0 (17/213)	5.0 (23/457)	10.3 (26/252)
Fab NIgG‖	None	0 (0/901)	0 (0/500)	0 (0/500)
Fab id	1.0 μl id serum¶	0.5 (3/602)	0.3 (1/593)	0.2 (1/503)
Fab id	5.0 μl normal A/J serum	6.3 (18/286)	3.8 (11/288)	9.6 (38/396)
Fab id	5.0 μl "suppressed" immune serum**	7.8 (85/1096)	3.9 (11/284)	10.0 (48/479)

* The mice were suppressed with respect to the CRI, then hyperimmunized with KLH-Ar as described in the text. They were allowed to rest for 9 wk before sacrifice. The spleens of 13 mice were pooled to carry out the experiments of Tables I, II, and III.

‡ Depleted of Ig-positive cells by the method of Parish et al. (see Materials and Methods). Data on sensitivity to anti-Thy-1.2 antibodies are given in Table II.

§ Fab fragments of anti-Ar antibodies possessing the CRI.

‖ Fab fragments of nonspecific A/J IgG.

¶ Containing 3.5 mg/ml of anti-Ar antibody possessing the CRI.

** Pooled serum from A/J mice, suppressed for the CRI then hyperimmunized. The serum contained 3.7 mg/ml of anti-Ar antibodies.

TABLE II

Sensitivity of Splenic Lymphocytes to Anti-Thy-1.2 Antiserum

Treatment of cells*	Spleen	T (nylon wool)	T(Ig^-)‡	T rosettes§
	% Killed (number of viable cells are in parentheses)			
Anti-Thy-1.2	0 (211/200)‖	20 (120/150)	0 (255/250)	0 (254/250)
C	0 (213/200)	0 (150/150)	9 (229/250)	8 (230/250)
Anti-Thy-1.2 + C	51 (98/200)	74 (39/150)	82 (46/250)	92 (19/250)

* See Materials and Methods.

‡ See footnote ‡, Table I.

§ Rosettes were prepared from T cells depleted of Ig-positive cells according to Parish et al. (Materials and Methods). The procedures used caused lysis of the RBC, so that the cells counted were free lymphocytes derived from the rosettes (see text).

‖ The values represent the number of cells in a given volume of the hemocytometer chamber, with or without treatment, respectively. The control tube contained only RPMI-1640 medium and fetal calf serum. Final volumes were the same in each test. In some instances the number of cells counted in the treated samples were up to 6% higher than in the controls, owing to experimental error in counting.

received no cells, or received RBC coated with the idiotype but no lymphocytes (groups 8 and 9), the titers of anti-Ar antibodies ranged from 0.17 to 1.1 mg/ml with a mean of 0.45 mg/ml. These values are typical of titers obtained in irradiated recipients, which produce considerably lower concentrations of anti-Ar antibody than nonirradiated mice. Within a group there was no apparent correlation between the titer of anti-Ar antibody and the concentration of CRI per unit weight of antibody.

Transfer of 1×10^7 T cells (group 1) resulted in suppression of the CRI although normal titers of anti-Ar antibodies were formed by the recipients. A mixture with RBC coated with Fab fragments possessing the CRI, but under

TABLE III

*Depletion of Suppressor Cells by Rosetting and Suppression by Rosettes**

Group no.	Cells transferred	No. of mice	Mean anti-Ar titer	Anti-Ar Ab required for 50% inhibition‡
			mg/ml	*ng*
1	10^7 T-enriched cells	5	1.3 (0.3–4.2)	3,000, >10,000, >25,000, >27,000, >105,000§
2	10^7 T-enriched cells + 2×10^8 RBC(id)‖	4	1.8 (0.7–3.8)	>17,500, >19,000, >52,000, >95,000
3	10^7 T-enriched cells depleted by rosetting¶	4	1.4 (0.2–5.1)	40, 69, 170, 200
4	5×10^5 T-enriched cells	9	0.5 (0.1–0.9)	56, 123, 125, 160, 260, 330, 700, 780, 840
5	5×10^5 T-enriched cells + 2×10^8 RBC(id)	4	1.0 (0.1–3.1)	160, 260, 370, 780
6	5×10^5 T-enriched cells as rosettes	9	1.8 (0.1–4.4)	4,200, >2,500, >22,500, >25,000, >25,000, >50,000, >57,500, >92,000, >118,000
7	5×10^5 T-enriched cells depleted by rosetting¶	10	0.4 (0.1–0.9)	25, 26, 31, 37, 38, 56, 63, 100, 130, 140
8	None	5	0.5 (0.1–1.1)	30, 47, 56, 56, 100
9	2×10^8 RBC(id)	5	0.4 (0.1–0.5)	17, 39, 42, 150, 163

* See footnote *, Table I, for a description of the source of donor cells. Rosettes were prepared with A/J RBC coated with Fab fragments of Anti-Ar antibodies possessing the CRI (see text). Recipient mice had received 200 R. Donors were suppressed with anti-id antiserum, hyperimmunized, and allowed to rest for 9 wk, as described in the text, before sacrifice.

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

§ Wherever the (>) symbol appears, 25 μl of serum was tested as inhibitor.

‖ RBC(id) refers to A/J RBC coated with Fab fragments of anti-Ar antibodies with CRI. In this group, the two types of cells were transferred as a mixture without centrifuging; i.e., not as rosettes.

¶ Using a 20:1 ratio of idiotype-coated RBC to lymphocytes.

conditions where rosettes did not form (i.e., without centrifuging to produce a pellet; group 2), had no effect on the suppressive activity of the T cells. However, when rosettes were allowed to form and were then removed by centrifugation (group 3), the remaining lymphocytes were not capable of suppressing the CRI. The adoptive transfer of 5×10^5 T cells did not induce the suppressed state (group 4), whereas transfer of the same number of rosette-forming T cells, as rosettes, was highly suppressive in each of the recipients (group 6). The adoptive transfer of 2×10^8 idiotype-coated RBC alone did not result in suppression.

Discussion

The experiments confirm our previous finding that substantial percentages of idiotype-specific rosette-forming lymphocytes can be produced under appropriate conditions (6, 7).[2] The system involves the anti-Ar antibodies of A/J mice, which exhibit strong intrastrain idiotypic cross-reactivity (1). The procedure consists, first, of suppressing the capacity to produce the CRI by administration of rabbit anti-id antiserum. Starting 2 wk later, the mice are hyperimmunized with KLH-Ar. High percentages of idiotype-specific rosette-forming T cells appear after a rest period of at least 6 wk; the percentage of RFC was much smaller when only 2 wk were allowed to elapse after the last inoculation of KLH-Ar (6, 7). Idiotype-specific RFC can also be induced in mice suppressed by adoptive transfer of lymphoid cells from suppressed syngeneic mice, rather than inoculation of anti-id antiserum (6).

Rosettes are formed with A/J RBC coated with Fab fragments of anti-Ar antibodies bearing the CRI. Evidence for idiotypic specificity included the failure to form rosettes with RBC coated with Fab fragments of nonspecific IgG; the inhibitory capacity of anti-Ar antiserum containing the CRI; and the absence of inhibition by anti-Ar antibodies lacking the CRI. Additional evidence,

reported earlier (6, 7),[2] is the inhibitory capacity of anti-id antibody, present in the reaction mixture; the failure of anti-mouse IgG to cause inhibition; and the inhibition by free haptens, *p*-aminophenylarsonate or (*p*-azobenzenearsonic acid)-*N*-acetyl-L-tyrosine. It was also found that the receptors on lymphocytes responsible for rosette formation are synthesized by the cells and not passively adsorbed, and that most or all of the lymphocytes forming rosettes are T cells (6, 7).[2] The latter observation was confirmed in the present study (Table II). The data in Table I also indicate that T cells prepared by passage through nylon wool or by selective removal of B cells (10) are both capable of forming idiotype-specific rosettes.

The principal new observation is that the idiotype-specific rosette-forming lymphocytes include the suppressor cells. This was demonstrated in two ways (Table III). After removal of the rosette-forming lymphocytes by centrifugation, those lymphocytes remaining in the supernate showed a greatly reduced capacity to suppress the CRI upon adoptive transfer into mildly irradiated recipients. This cannot be attributed simply to the presence of idiotype-coated RBC in the mixture since the latter did not have suppressive activity (group 9, Table III). In addition, a mixture of 5×10^5 T cells plus RBC, prepared under conditions that do not permit rosette formation, was not suppressive, whereas the same number of rosetted cells caused suppression of the CRI after adoptive transfer (groups 5 and 6). Thus, the rosettes themselves were highly suppressive. The results therefore indicate that the rosette-forming T cells include the suppressor population. They do not, however, prove that all of the rosette-forming T cells are suppressor cells. This question is under investigation.

Another point of interest is the mechanism of formation of such large numbers of idiotype-specific T cells. We have previously shown[2] that injection of anti-id antiserum without subsequent hyperimmunization results in the formation of very small numbers of rosette-forming lymphocytes. The requirement for antigenic stimulation suggests that the agent which stimulates the multiplication of idiotype-specific suppressor T cells may be a complex of the idiotype with antigen. This possibility has been suggested by Eichmann (4). Although the mice were suppressed with respect to formation of the CRI, small amounts of idiotype might be produced which immediately form complexes with the excess of antigen present during hyperimmunization.

The fact that the suppressor cells interact with RBC coated with the idiotype suggests the possibility that T-cell-mediated suppression of idiotype occurs as a result of direct interaction between suppressor T cells and B lymphocytes bearing receptors with the CRI. This interpretation must be presented with some caution in view of the observation that allotype-specific suppression is mediated by T cells which interact with other T cells (16). The ability to generate large numbers of idiotype-specific T cells, and to isolate the suppressors, should facilitate the investigation of the mechanisms of induction of these cells and of suppression.

Summary

All A/J mice produce anti-*p*-azophenylarsonate (anti-Ar) antibodies, some of which share a cross-reactive idiotype. The idiotype can be suppressed by treat-

ment with anti-idiotypic antiserum before immunization, although normal concentrations of anti-Ar antibodies are synthesized. We have previously reported that such suppressed mice, if hyperimmunized and then allowed to rest, contain up to 10% of splenic T cells which form rosettes with autologous RBC coated with Fab fragments of anti-Ar antibodies bearing the idiotype. Our present results indicate that the rosette-forming T cells include the idiotype-specific suppressor T-cell population. The suppressive activity is largely depleted by removal of the rosette-forming lymphocytes, and the rosettes themselves are highly suppressive. The data do not establish whether all of the idiotype-specific rosette-forming cells are suppressor cells. The system may provide a source of large numbers of suppressor cells for further study, and facilitate investigation of the mechanism of generation of idiotype-specific suppressor cells.

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