NYLON ADHERENT ANTIGEN-SPECIFIC ROSETTE-FORMING T CELLS*

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Both B and T lymphocytes interact with antigens in a highly specific fashion. The most direct demonstration of lymphocyte recognition of antigen has been the in vitro demonstration of antigen-binding cells obtained from immune and nonimmune individuals. However, while antigen-binding B cells can be demonstrated readily, direct visualization of antigen-binding T cells has been more problematic (1). Binding of soluble antigens by T cells has been particularly difficult to demonstrate and was thought to be due to a low site density of antigen-specific receptors in the T-cell membrane (1, 2). It is more likely however, that the difficulty in demonstrating binding of soluble antigen by T cells is due to particular temperature and antigen concentration requirements requisite for antigen binding to occur (3, 4).

More success in demonstrating antigen-binding T cells in immune or nonimmune animals has been achieved when T cells bind antigen in solid phase; either soluble antigen linked to fibers (5) or cell membrane-associated antigens (6–8). However, immune T cells which bind heterologous erythrocytes (T-rosette-forming cell, T-RFC)[1] differ from their B-cell counterparts in that they are highly unstable and dissociate rapidly in vitro (9). These observations suggest that the avidity of T-cell antigen receptors is lower than B-cell receptors and/or the physiology of the lymphocyte antigen interaction is different for T and B cells.

In the present study we sought to examine in detail the requirements for the generation of immune rosette-forming T cells so that functional characterization of these cells might be attained. It has previously been suggested that some T-RFC function as effector cells for delayed-tape hypersensitivity to sheep erythrocytes (SRBC) (10). Our results indicate that T-RFC belong to a distinct minor subset of T cells which adhere to nylon wool. Although some of the T-RFC may be involved in delayed-type hypersensitivity (DTH) reactions, the function of the majority of T-RFC remains unknown. However, there are a number of

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[1] *Abbreviations used in this paper:* B-RFC, nonthymic-derived lymphocytes-forming rosettes; DPBS, Dulbecco's phosphate-buffered saline; DTH, delayed-type hypersensitivity; FCS, fetal calf serum; HRBC, horse erythrocytes; RBC, erythrocytes; RFC, rosette-forming cells; SRBC, sheep erythrocytes; T-RFC, thymic-derived lymphocytes-forming rosettes.

similarities between T-RFC and two interacting regulatory T-cell subclasses recently described by Tada (11), and we suggest they may indeed be the same cells.

Materials and Methods

Mice. Mice were 6–8 wk old male BDF_1 (C57B1/6 × DBA/2) from The Jackson Laboratory, Bar Harbor, Maine. They were rested 1 wk in our animal facilities before use.

Immunization and Cyclophosphamide Treatment. Mice were anesthetized with ether and immunized by intravenous injection of 0.2 ml freshly washed SRBC or horse erythrocytes (HRBC) (Colorado Serum Co., Denver, Colo.) in sterile pyrogen-free saline (Abbott Laboratories, North Chicago, Ill.). Unimmunized controls received intravenous injections of saline. In some experiments groups of mice received an intraperitoneal injection of freshly prepared cyclophosphamide (Mead Johnson & Co., Evansville, Ind.) in saline. Two doses were employed: 20 mg/kg 1 day before immunization or 200 mg/kg 2 days before immunization (12).

Elicitation of DTH. 4 days after immunization, mice (five to six/group) were injected in the ventral footpad with 0.03 ml of 20% erythrocytes (RBC). Footpad thickness was measured with a micrometer before testing and at 24 and 48 h as previously described (12). The percent increase in footpad thickness was calculated and the Student's t test was used to compute the statistical significance ($P < 0.05$) between groups. Recipients of spleen cells from immunized donors were tested with 50% RBC.

Preparation of Cell Suspensions. Spleens of mice were removed into chilled Dulbecco's calcium, magnesium-free, phosphate-buffered saline (DPBS) pH 7.2 or Hank's balanced salt solution and were disrupted by either three strokes in a loose fitting tissue grinder or by pressing the spleens between two frosted glass microscope slides. Erythrocytes were removed by either hypotonic lysis for 5 s in distilled water followed by washing in DPBS or by lysis with 0.83% NH_4Cl. Cellular debris was removed by layering the cells over 0.5 ml fetal calf serum (FCS) and incubation for 5 min at 4°C. The buffer layer containing the cells was washed three times in cold DPBS before use. In most experiments spleens from each mouse in a group (two to three/group) were pooled and rosette assays were done on the pooled suspensions. In transfer experiments, 4 days after donor immunization, cells were harvested in medium RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) containing penicillin, streptomycin, and 5% FCS. After washing, various doses of cells were transferred intravenously in 0.5 ml medium and recipients were footpad tested the following day.

Separation of Nylon Adherent and Nonadherent Cells. The nylon column method of Julius et al. (13) was employed. The nylon was prepared as previously described (14) and 1 ml of spleen cell suspension was applied to the column per 0.5 g of nylon at a concentration of 5×10^7/ml in media RPMI 1640 containing 5% FCS. After incubation at 37°C × 45 min the nonadherent cells were eluted with fresh 37°C media and then adherent cells were recovered by agitating the nylon. In general, 80% of the cells applied were recovered; they were >95% viable by dye exclusion test, and nonadherent cells accounted for 15–20% of recovered cells. Nylon nonadherent cells were considered to be a subpopulation of relatively pure T cells as >95% were killed by anti-Thy-1 serum and complement and <2% had surface immunoglobulin as detected by an indirect fluorescent anti-immunoglobulin assay (14).

Treatment with Anti-Thy 1.1 Serum and Complement. Splenic lymphoid cells were suspended in DPBS at a concentration of 2×10^7 cells/ml. AKR anti-C_3H Thy 1.1 serum was added to a final concentration of 1:20 and the cells were incubated for 30 min at 4°C. The cells were then washed once, resuspended in medium RPMI 1640 to 2×10^7 cells/ml, and guinea pig serum was added as a source of complement to a final concentration of 1:30. The cells were incubated for 1 h at 37°C and then washed two times with chilled DPBS. Normal AKR serum served as a control in these experiments. The percent of rosette-forming cells (RFC) sensitive to treatment with anti-Thy 1.1 + complement was calculated as:

$$\frac{\text{total no. RFC recovered after treatment with anti-Thy 1.1 + complement}}{\text{total no. RFC recovered after treatment with normal AKR serum}} \times 100$$

Rosette Assay. Two methods were used for the enumeration of rosette-forming cells (RFC).

CENTRIFUGATION ASSAY. A modification of the method of Elliot and Haskill (9) was employed. Spleen cells were suspended in 1 ml DPBS at a concentration of 2×10^7 cells/ml. SRBC or HRBC (0.05 ml of a 10% suspension) were added and the mixture was centrifuged at 4,000 rpm for 3 min in 10 × 75 mm round-bottomed tubes at 4°C in a Sorvall RC-5 centrifuge (Sorvall, Ivan, Inc., Norwalk, Conn.). Supernates were removed, the pellets were held at 4°C for 30–45 min, and then were resuspended to 2×10^7/ml in DPBS which contained 0.1% (vol/vol) glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.). This method was optimal for demonstrating thymus-derived RFC (T-RFC).

INCUBATION METHOD. The method of Zaalberg et al. (15) was employed. Spleen cells were suspended in DPBS to a concentration of 10^7 cells/ml. SRBC or HRBC (0.1 ml of a 10% suspension) was added and the mixture was incubated (in 10 × 75 mm round bottom tubes) for 1 h at 37°C at a 45° angle. The tubes were then transferred to a 4°C refrigerator, held overnight, and RFC were enumerated the following day. RFC prepared by centrifugation were resuspended gently with a Pasteur pipette. RFC prepared by incubation were resuspended by gently rolling the tubes. RFC were enumerated in an improved Neubauer hemocytometer (American Optical Corp., Buffalo, N.Y.) at ×200 magnification. The entire chamber was scanned (approximately 18,000 cells). Nucleated cells binding more than four erythrocytes were scored as a RFC.

Results

Effect of Glutaraldehyde on Rosette-Forming Cells. Our preliminary experiments were designed to enumerate RFC in spleens of mice receiving an intravenous low dose of SRBC (0.01%, about 2×10^5 RBC) that was known to be optimal for the induction of DTH (16–18). At this antigen dose it seemed likely that elevated numbers of T-RFC would be detected. RFC were prepared by centrifugation and resuspension in DPBS alone. In some cases a three to fivefold increase in RFC above that found in control mice was observed, but the results were quite variable. Moreover, if the formed RFC were held in suspension for as short a time as 10 min at 4°C the number of RFC decreased by 50–80%. In contrast, mice which were immunized i.v. with 10% SRBC had weak DTH responses and stable RFC.

Rosettes in mice receiving optimal intravenous immunizing doses for DTH were found to be stabilized by fixation with glutaraldehyde, as described by Elliot and Haskill (9). Pellets containing rosettes were resuspended in DPBS containing various concentrations of glutaraldehyde and RFC were enumerated after incubation for 5 min at 4°C. As shown in Fig. 1 only a modest increase in RFC above background was observed when RFC were prepared without glutaraldehyde (1,100 RFC/10^6 spleen cells from mice immunized with 0.2 ml of 0.01% SRBC vs 700 RFC/10^6 in mice receiving saline). Inclusion of glutaraldehyde with DPBS resulted in a significant increase in detection of RFC in the immune cell population, 0.2–2.0% glutaraldehyde being optimal. Moreover, glutaraldehyde-fixed rosettes did not deteriorate when incubated for 24 h at 4°C. In contrast, glutaraldehyde fixation caused no significant increase in the background RFC in spleens from unimmunized mice (Fig. 1) or in mice receiving 10% SRBC i.v. (data not shown). Spleen cells incubated with glutaraldehyde for 10 min at 4°C before centrifugation with RBC formed no RFC.

Antigen Specificity of RFC in Mice Receiving Intravenous Immunization with Low Doses of RBC. The results presented above indicate that the spleens of mice receiving 0.01% SRBC contain elevated numbers of cells capable of binding SRBC 4 days after immunization. The antigen-binding specificity of these cells was determined by immunizing groups of mice with either 0.01%

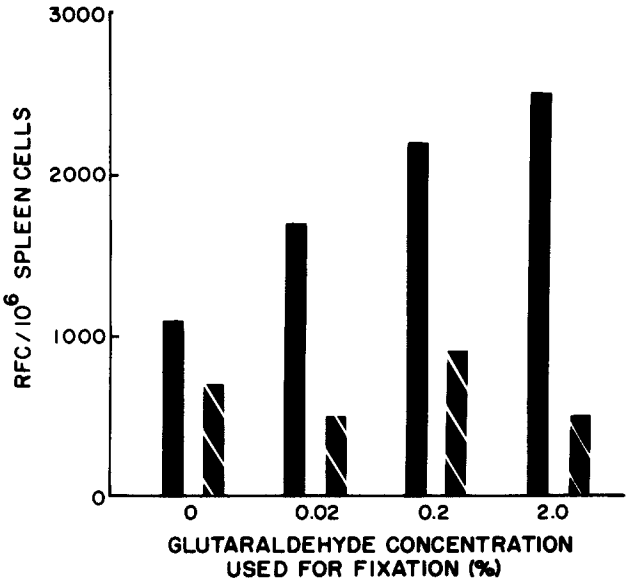

FIG. 1. Stabilization of rosette formation by glutaraldehyde. Spleen cells obtained 4 days after immunization of mice with 0.01% SRBC were mixed with SRBC and allowed to form rosettes. After incubation at 4°C the cells were resuspended in various concentrations of glutaraldehyde in DPBS and RFC were enumerated. Closed; RFC in immunized mice. hatched bar; RFC in mice receiving saline only.

TABLE I
Antigen Specificity of RFC in Mice Immunized Intravenously with Low Doses of RBC

Immunization	RFC/spleen	
	SRBC	HRBC
Saline	5,000	8,000
0.01% SRBC	17,000	1,500
0.01% HRBC	3,900	86,000

Spleens were removed from three mice in each group 4 days after immunization and pooled for the preparation of cell suspensions used in the rosette assay.

SRBC or HRBC. 4 days later spleen cells were assayed for RFC by using both SRBC and HRBC in each group. Immunization with SRBC elevated splenic RFC over threefold from a background of 5,000/spleen to 17,000/spleen but did not raise the numbers of cells binding HRBC (Table I). In fact, the number of HRBC-binding cells was reduced from 8,000/spleen to 1,500/spleen. Immunization with HRBC raised the number of HRBC-binding cells approximately 10-fold but had no effect on RFC-binding SRBC. The ability of HRBC to elicit specific RFC at low antigen doses was found to be consistently better than SRBC. The majority of RFC detected at this and lower doses of either HRBC or SRBC were T cells (see below).

Effect of Antigen Dose on Rosette-Forming Cells. We next examined in

detail the relationship between antigen dose and the number and type of RFC elicited. Mice received from 2×10^3 to 2×10^8 SRBC i.v. and spleens were harvested 5 days later. Portions of the cell suspensions were treated with anti-thy 1.1 serum and complement and antiserum-treated and untreated cell populations were assayed for RFC by both centrifugation and incubation assays. The number of T-RFC was obtained by multiplication of the total number of RFC in the untreated cell populations by the percent of RFC eliminated by anti-Thy 1.1 treatment. The remainder were considered B-RFC.

As shown in Fig. 2 A, no rise in centrifuged total RFC-binding SRBC was evident until administration of at least 0.01% (2×10^5) SRBC. At higher immunizing doses the number of RFC rose rapidly reaching a peak at 1% (2×10^7) SRBC. However, when mice were immunized with low doses of RBC and T and B-RFC were enumerated separately, changes in the numbers of these subsets of RFC (relative to background levels) were observed. Thus, the T-RFC levels in mice receiving saline were usually only approximately 15–20% of the total number of background RFC. As shown in Fig. 2 A the number of T-RFC rose approximately threefold with injection of as few as 2×10^3 SRBC (0.0001%). The number of T-RFC remained constant up to administration of 0.01% SRBC and comprised at least 60% of the total RFC population. The number of T-RFC rose again and plateaued when (0.1–1.0%) SRBC were injected. However, 10% SRBC caused an 80% reduction in the number of T-RFC from levels attained with 1% SRBC. In contrast, injection of 0.0001% SRBC resulted in a 44% reduction in the number of B-RFC relative to background levels. The number of B-RFC began to rise from this level (9,000/spleen) at 0.01% SRBC and continued to rise until 1.0% SRBC where the levels were approximately sixfold higher than T-RFC.

Substantially similar results were obtained when mice were injected with HRBC. However, as shown in Fig. 2 B the increase in total RFC began at 2×10^4 HRBC (0.001%) and peaked at 0.1% erythrocytes. T-RFC began to increase at 2×10^3 HRBC and peaked at 2×10^6 HRBC. Higher doses of HRBC (more than 2×10^6) resulted in substantial decreases in T-RFC levels. Similarly, B-RFC levels fell to 25% of background levels at low immunizing doses (2×10^3 HRBC) and did not rise significantly until 2×10^5 HRBC were injected. B-RFC levels peaked at 2×10^7 HRBC and fell somewhat at 2×10^8 HRBC.

In contrast to the results obtained when RFC were prepared by centrifugation, RFC assessed by the incubation assay presented a markedly different picture (Fig. 2 C). At no point in the dose-response curve were significant numbers of T-RFC observed by the incubation assay and consequently only total RFC are shown. As was observed when B-RFC were detected by the centrifugation technique the number of B-RFC detected by incubation fell dramatically from background levels when 0.0001–0.01% SRBC were injected. Elevated numbers of RFC above normal background levels were observed when 0.1% SRBC were injected and peak levels were obtained with 1–10% SRBC.

The results presented above indicate that the antigen dose requirements for the generation of T and B-RFC are markedly different. The population dynamics of T and B-RFC relative to antigen dose and regardless of absolute numbers is shown in Fig. 3. It can be seen that with very low dose immunization, T-RFC are selectively generated and B-RFC are suppressed. This is particularly

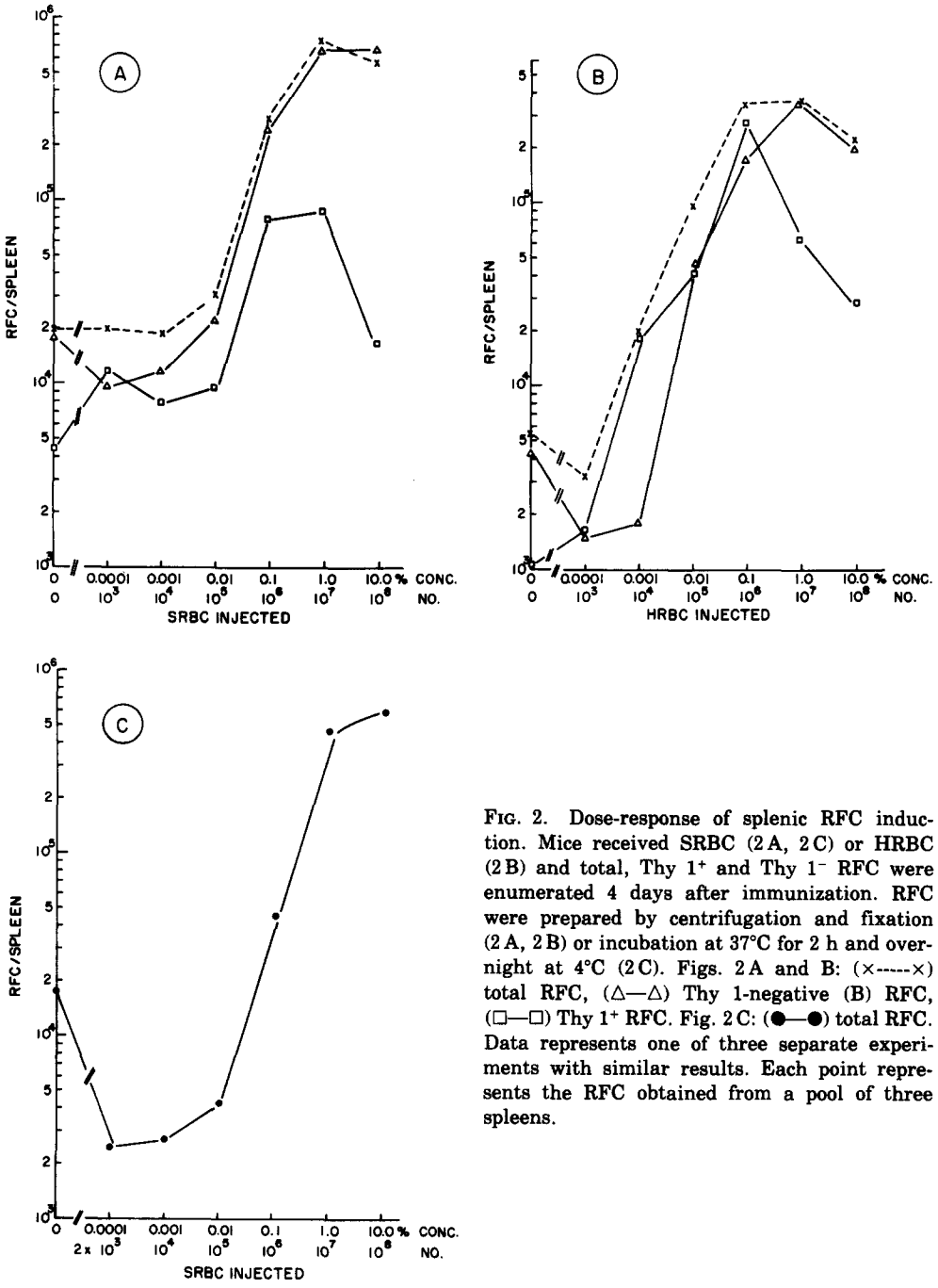

FIG. 2. Dose-response of splenic RFC induction. Mice received SRBC (2A, 2C) or HRBC (2B) and total, Thy 1^+ and Thy 1^- RFC were enumerated 4 days after immunization. RFC were prepared by centrifugation and fixation (2A, 2B) or incubation at 37°C for 2 h and overnight at 4°C (2C). Figs. 2A and B: (×-----×) total RFC, (△—△) Thy 1-negative (B) RFC, (□—□) Thy 1^+ RFC. Fig. 2C: (●—●) total RFC. Data represents one of three separate experiments with similar results. Each point represents the RFC obtained from a pool of three spleens.

evident when $2 \times 10^3 - 2 \times 10^4$ HRBC are administered. The proportion of T-RFC in the spleen shifts from 15–20% background levels to more than 90%. As the dose of antigen is increased, B-RFC are generated such that the proportion of T-RFC vs. B-RFC becomes reversed at high antigen doses.

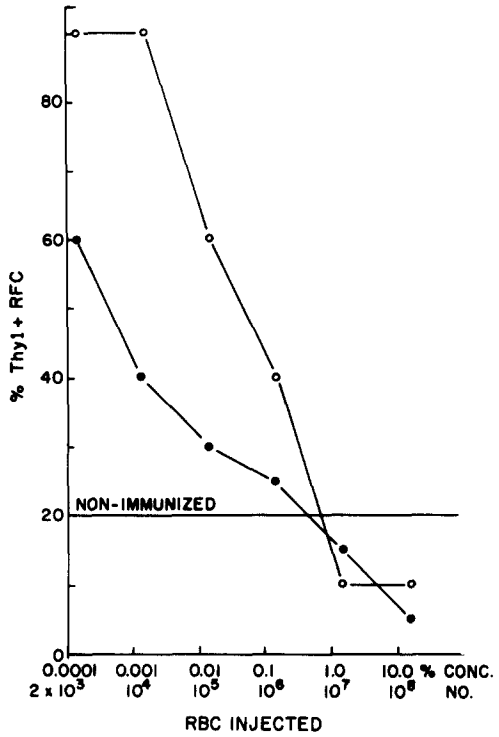

FIG. 3. Percent Thy 1^+ splenic RFC as a function of antigen dose. Mice were immunized with SRBC or HRBC and spleens were assayed for RFC 4 days after immunization. Thy 1^+ RFC were assessed by determining the percent of RFC reduction when spleen cells were treated with anti-Thy 1 antiserum and C′ before rosette formation. (●—●) TRFC to SRBC, (○—○) TRFC to HRBC. Data represents the results obtained in three separate experiments.

Fractionation of T-RFC and B-RFC on Nylon Wool Columns. From the above it is apparent that immunization of mice with low doses of erythrocytes selectively induces the generation of T-RFC. To document this point further splenic lymphocytes from immune and nonimmune mice were applied to nylon wool columns to separate nylon adherent and nonadherent cells (13). In general, approximately 15–20% of the cells were nonadherent and these were 95–98% T cells. However, as shown in Table II the RFC population was almost completely adherent to nylon regardless of the dose of antigen used for immunization. Thus, in 0.01% SRBC-immune mice 70% of the RFC were T cells yet nearly all of these adhered to nylon. When mice received 10% SRBC, only 15% of the total RFC population were T cells, but 91% of these T-RFC were nylon adherent. In this case the majority of adherent RFC were B cells. In these experiments we also enumerated RFC which were induced in mice which received 10% SRBC and 200 mg/kg cyclophosphamide 2 days before injection of antigen. In such mice antibody production is abrogated and DTH is elicited (12). In cyclophosphamide-treated mice 81% of the RFC were T cells and 98% of these T-RFC adhered to nylon. It should be noted that the total number of T-RFC in cyclophosphamide-treated mice immunized with 10%

TABLE II
Adherence of T-RFC and B-RFC to Nylon Wool

SRBC immunization	Cyclophosphamide pretreatment, 200 mg/kg	Total RFC Thy 1+	Nylon adherent		Nylon nonadherent	
			Total recovered	Thy 1+	Total recovered	Thy 1+
		%	%		%	
0.01%	–	70	92	66	8	97
10%	–	15	91	NT*	9	NT
10%	+	81	98	88	2	100
Saline	–	20	99	19	1	100

Data represents the pooled results obtained in three separate experiments.
* NT, not tested.

SRBC was not significantly different from that obtained just by immunization with 0.01% SRBC. Similar results to those presented in Table II were obtained when HRBC were utilized as antigen (data not shown).

Development of RFC and Delayed Hypersensitivity. In addition to assessing the development of T and B-RFC as a function of antigen dose, we tested concurrently the development of DTH to the immunizing antigen. Thus, 4 days after immunization some mice were assayed for RFC and others were challenged in the footpad with erythrocytes, and swelling was measured 24 h later. The results shown in Fig. 4 demonstrate that maximum footpad reactions were obtained with 10^4-SRBC and 10^5-HRBC where T-RFC were selectively induced relative to B-RFC. At antigen doses yielding maximum numbers of B-RFC, (see Figs. 2 A and B) delayed hypersensitivity was reduced or absent. Although the increase in T-RFC paralleled the increase in delayed hypersensitivity between 10^3 and 10^5 erythrocytes, peak numbers of T-RFC were however, observed at antigen doses that resulted in reduced-delayed hypersensitivity. There is the suggestion of a shoulder in the dose-response curve for T-RFC at the antigen dose resulting in maximum-delayed hypersensitivity indicating a possible biphasic nature to the dose-response curve. This is particularly evident for SRBC (Fig. 4 A).

Effect of Cyclophosphamide Pretreatment on the Generation of T and B-RFC. Previous findings (16–18) have shown that mice immunized intravenously with 0.01% RBC have optimal DTH responses and no hemagglutinating antibody responses at day 4. Mice receiving higher immunizing doses have progressively diminished DTH reactions and progressively increased antibody responses. DTH can be augmented in these latter mice by pretreatment with cyclophosphamide in high doses (200 mg/kg), which are B-cell depleting, or in low doses (20 mg/kg), which do not affect antibody responses (12). Since high doses of cyclophosphamide will facilitate T-RFC formation in mice receiving 10% SRBC (Table II), we next compared the total RFC and the T and B-RFC responses in controls vs. mice pretreated with 20 mg/kg cyclophosphamide and immunized with various doses of HRBC. The continuous rise in B-RFC and T-RFC over the immunizing dose range of 10^3–10^6 HRBC was similar in control vs. cyclophosphamide-treated mice. However, as shown in Fig. 5, the significant decline in T-RFC at the highest immunizing dose (10^7, 1% RBC) was prevented

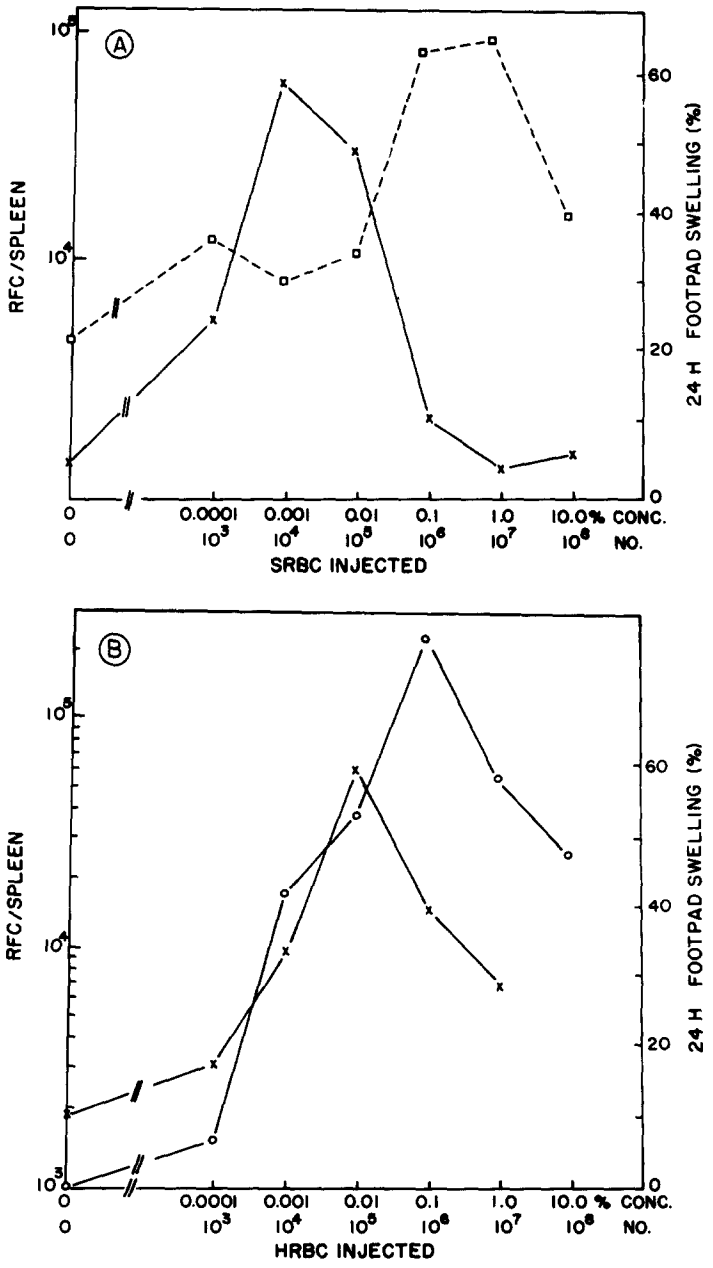

FIG. 4. Dose-response of Thy 1^+ RFC and elicitation of DTH. Mice received SRBC (4A) or HRBC (4B) 4 days before either enumeration of RFC or footpad challenge with erythrocytes. Fig. 4A: (□—□), Thy 1^+ RFC, (×—×) 24 h footpad swelling, percent increase. Fig. 4B: (○—○) Thy 1^+ RFC, (×—×) 24 h footpad swelling, percent increase. RFC results are the number of RFC enumerated in a pool of three spleens. Footpad swelling represents the average increment in footpad thickness of five mice/group.

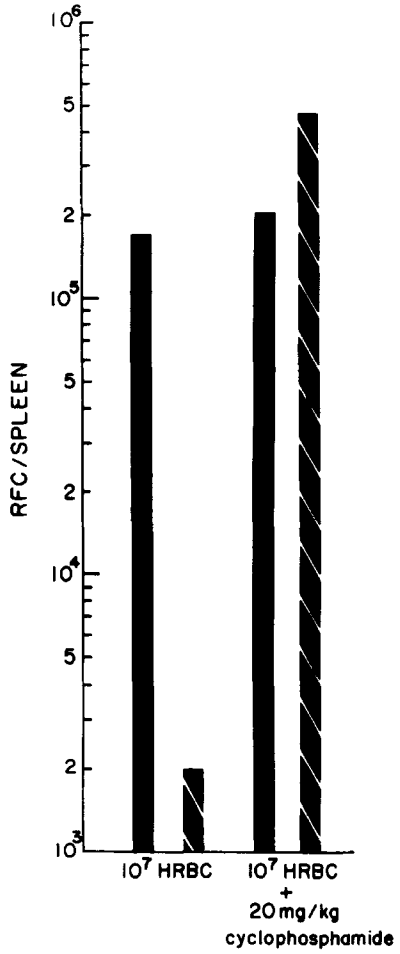

FIG. 5. Effect of cyclophosphamide treatment on the induction of Thy 1^+ and Thy 1-negative RFC in high antigen dose-immune mice. Mice received 20 mg/kg cyclophosphamide 1 day before immunization with 1×10^7 HRBC. 4 days after injection of HRBC spleens were harvested and Thy 1^+ and Thy 1-negative RFC were enumerated. Hatched bar; Thy-1-positive. Closed bar; Thy 1-negative RFC. Data are representative of one of two experiments with similar results.

by cyclophosphamide pretreatment. Thus, 20 mg/kg cyclophosphamide did not affect B-cell responses over immunizing doses that were optimal or supraoptimal for DTH. However, at the highest immunizing dose, 20 mg/kg cyclophosphamide affected T-cell responses. DTH was augmented and the decline in T-RFC was abolished.

Nylon Wool Column Fractionation of Spleen Cells Transferring DTH. Although nylon nonadherent cells are a relatively pure subpopulation of T cells, the results of the previous section indicate that antigen-specific T-RFC are largely adherent to nylon wool. The separation of these T-cell subsets by nylon adherence thus affords a means to determine the capacity of these subsets to transfer DTH. Table III shows one of three similar experiments in

TABLE III
Transfer of DTH with Immune Spleen Cells Fractionated on Nylon Wool Columns

Transferred spleen cell type	No. cells transferred/mouse	24 h footpad swelling ± SE
		%
Whole	40×10^6	33 ± 1
Whole	20×10^6	25 ± 3
Whole	10×10^6	20 ± 4
Whole	5×10^6	17 ± 2
Nylon adherent	20×10^6	31 ± 3
Nylon adherent	10×10^6	22 ± 1
Nylon adherent	5×10^6	21 ± 2
Nylon adherent	2.5×10^6	17 ± 1
Nylon adherent	1.25×10^6	18 ± 1
Nylon nonadherent	40×10^6	35 ± 3
Nylon nonadherent	20×10^6	25 ± 2
Nylon nonadherent	10×10^6	26 ± 5
Nylon nonadherent	5×10^6	20 ± 2
Nylon nonadherent	2.5×10^6	18 ± 1
Nylon nonadherent	1.25×10^6	20 ± 4
	Nil	9 ± 3

Donors were immunized with 0.01% SRBC and cells were harvested and transferred at day 4.
The following day recipients (five/group) and a control group were footpad tested with 50% SRBC. Significant responses were elicited in all recipients.
% = percent increase in footpad thickness relative to unchallenged mice.

which whole spleen cells, nylon nonadherent cells, and nylon adherent cells were all able to transfer DTH. Adherent or nonadherent cells were equivalent in the ability to transfer DTH when tested in a limiting dilution assay. In both instances only 1.25×10^6 cells from donors immunized just 4 days previously with only 2×10^5 SRBC were competent to transfer DTH. This represented about 2% of the cells in a donor spleen. For both adherent and nonadherent cells there was a broad plateau in the dose-response curve so the 8-fold range from 1.25-10 $\times 10^6$ cells produced equivalent transfers of DTH. It was concluded that nylon adherent and nonadherent cells could transfer DTH in small numbers and that in either case cells containing very few antigen-specific T-RFC were effective in systemic transfer of DTH.

Discussion

We have found that antigen-specific rosette-forming T cells can be demonstrated in mice immunized with a wide dose-range of sheep or horse erythrocytes. The T-RFC induced by erythrocyte immunization differ from B-RFC in the stability of lymphocyte-RBC interaction and in dose-response requirements. We found, in confirmation of the observations of Elliot and Haskill (9), that T-

RFC are highly unstable. Once T-RFC are formed they will deteriorate within 10 min of suspension unless fixed with glutaraldehyde. In fact, incubation of T cells with SRBC under B-cell rosetting conditions (19) results in complete loss of T-RFC. Moreover, incubation of splenic lymphocytes with SRBC for 15 min in suspension before centrifugation will result in a similar disappearance of T-RFC. These results suggest that T-cell erythrocyte interactions in suspension may result in some active process which releases the erythrocyte from the cell surface, perhaps with shedding or internalization of the antigen receptor. In support of this contention Elliot and Haskill (9) have shown that suspension of T rosettes in buffers containing sodium azide stabilizes rosette formation. However, pretreatment of lymphocytes with glutaraldehyde before addition of erythrocytes abrogates the formation of T-RFC. Such treatment may cross-link the antigen receptor at the cell surface or fix the cell surface in such a way as to either prevent antigen binding or membrane movement required for rosette formation.

The dose requirements for the induction of T and B-RFC were found to be distinctive at both low and high antigen doses. At extremely low doses of antigen, T-RFC are selectively induced. The dose of antigen required for B-RFC induction is at least 10-fold higher than that required for T-RFC. Moreover, at antigen doses resulting in selective T-RFC induction, B-RFC are suppressed below normal background levels. This reciprocal rise in T-RFC and fall in B-RFC at low antigen doses can make it appear as if no increase in RFC has occurred unless T and B-RFC are enumerated separately. The decrease in background B-RFC at low antigen doses may be nonspecific as suggested by the data in Table I. Immunization with 0.01% SRBC caused elevated RFC to SRBC, but this was accompanied by a profound decrease in background RFC to HRBC. Above the minimum B-RFC-inducing dose, B-RFC increase in linear fashion with log increases in SRBC. In contrast, the generation of T-RFC suggests a biphasic response with an increase in T-RFC between 0.001 and 0.01%, a plateau or shoulder between 0.01 and 0.1%, and a further increase between 0.1 and 1%. It is possible that the biphasic nature of the dose-response curve reflects differing optimal doses for the generation of two separate T-cell populations.

The functional role, if any, these T-RFC may have in the immune response is unknown. Our results indicate that they are not essential for the induction or elicitation of DTH response because: (*a*) maximum TRFC responses are found after immunization with doses of antigen which are 1–2 logs greater than those which produce optimal-delayed hypersensitivity responses and (*b*) DTH responses can be transferred with cells which pass through nylon columns, which are almost totally devoid of T-RFC. However, DTH responses can also be transferred with cells eluted from nylon columns. Since these cells contain T-RFC, it is possible that T-RFC are a second type of DTH inducer or effector cell. Alternatively T-RFC may act in concert with other T cells in effecting or regulating DTH responses.

While it is possible that T-RFC may play some regulatory role in the immune response, it is clear that they are not essential for T-cell-mediated suppression. This conclusion is based on the observation that the production of

T-RFC is markedly inhibited by high antigen doses which are known to be efficacious in producing suppressor T cells (20). In addition, the inhibiting effect of high antigen doses on T-RFC production is eliminated by pretreatment with cyclophosphamide, indicating that T-RFC as well as DTH effector cells are under suppressor cell control. Furthermore, we have been able to show significant suppressor activity in nylon passed T-cell populations from animals immunized with high doses of SRBC (20).

It is also clear that the T-RFC are not essential for T-cell-mediated helper effects because helper cells also pass through nylon columns. Thus, we are left with what appears to be an important T-cell population in that large numbers of T-RFC are formed after immunization, yet no specific immunological function can be assigned to them. However, since there are a number of similarities between T-RFC and certain T-cell subpopulations described by Tada (11), it is possible that the two cell types are either identical or closely related.

Tada (11) has described an Ly 2,3 T cell which acts to induce suppressor cells from the Ly 1,2,3, IJ-positive T-cell pool. Tada finds that both inducer cell and its target acceptor cell adhere to nylon. It should be noted that our preliminary evidence indicates that all T-RFC can be killed with an anti-Ly 2 antiserum and a variable proportion are killed with an anti-Ly 1, indicating that like Tada's two cells, our T-RFC are of the Ly 2,3 and Ly 1,2,3 phenotype. Thus, the T-RFC we have described may be cells involved in the fine tuning of the immune response. Our antigen dose-response studies also suggest the existence of two T-RFC subpopulations, one of which is activated at very low doses of antigen and another which appears at higher doses just before inhibition of T-RFC occurs. It would be interesting if the first population represented the Ly 2,3 inducer which at the higher doses recruited or induced the Ly 1,2,3 acceptor. The absence of T-RFC found at the higher doses could then be explained by the amplified suppression produced by the interaction of the two aforementioned subpopulations.

Tada has also shown that an antigen-specific factor passes from the suppressor inducer cell to its target, and is probably responsible for the activation of the target cell (11). It could be that the receptors on the T-RFC which bind the SRBC are the same or related molecules to the ones Tada has used to induce suppression. It is also possible that since a significant number of Fc positive T cells adhere to nylon (21) that the antigen-specific molecule is not necessarily made by the T-RFC but is demonstrable only on the cells which receive it passively. This could explain why it has been so difficult to demonstrate antigen-binding helper cells (10). They may shed or secrete their receptors too rapidly or too easily to be detected, while only the cells which passively pick up these factors or receptors can be demonstrated by antigen binding.

This raises the question of which cell is making the antigen-specific receptor on the T-RFC. Although it is possible that T-RFC make their own receptors, since T-RFC are found in the fraction of T cells which is rich in Fc-positive cells, one must at least consider the possibility that the factor-bearing cells do not make their own receptor. Our results clearly rule out the possibility that the antigen-binding receptor is made by a standard B cell. Thus, a significant increase in T-RFC can be produced with doses of antigen which are too low to

stimulate any measurable B-cell activity and doses of antigen inducing maximum B-RFC suppress T-RFC. In addition, we showed that at high doses of antigen, the production of T-RFC is markedly augmented by pretreatment with doses of cyclophosphamide which eliminate the production of B-RFC. Further, immunization with the low doses of antigen increase T-RFC and suppress B-RFC. Finally, no T-RFC are detected under B-cell rosetting conditions where secretion of Ig might be expected. These results do not rule out the possibility that there is a specialized subpopulation of B cells which make antigen-specific factors which are accepted by T-RFC. Alternatively, as we have suggested above, the T-RFC may passively acquire their specificity from products made by other T cells. We are presently testing some of the hypotheses we have offered above.

Summary

Shortly after intravenous immunization of mice with heterologous erythrocytes (RBC) antigen-specific Thy 1^+ cells which form rosettes with the immunizing RBC (thymic-derived lymphocytes-forming rosettes [T-RFC]) appear in the spleen. These T-RFC are much less stable than Thy 1^- RFC (non-thymic-derived [B-RFC]) although most if not all of both classes of RFC adhere to nylon. T-RFC are induced with low doses of antigen (which fail to induce B-RFC) and are inhibited by higher antigen doses which are optimal for induction of B-RFC. Pretreatment of mice with cyclophosphamide prevents the high dose inhibition of T-RFC. Although there are many parallels between the production of T-RFC and delayed-type hypersensitivity (DTH) it is unlikely that the T-RFC are essential for DTH reactions since DTH can be transferred with cells which pass through nylon, and such cells are almost totally depleted of T-RFC. Thus immunization can lead to the production of large numbers of antigen-specific T-RFC whose functional role in the immune response is unknown. However, the characteristics of the T-RFC suggest that they may play an important role in amplification of suppressor cell activity.

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