ROSETTE-FORMING ABILITY OF THYMUS-DERIVED LYMPHOCYTES IN CELL-MEDIATED IMMUNITY

I. Delayed Hypersensitivity and In Vitro Cytotoxicity*

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Specific recognition of antigen by thymus-derived (T) effector cells in delayed hypersensitivity response (DHR)[1] in vivo (1), and cell-mediated cytotoxicity in vitro (2–4), implies that these cells have specific receptors for antigen. Cytotoxic T cells can adhere specifically to allogeneic (2) and xenogeneic (4) target cell monolayers; and both precursor and effector T cells in delayed hypersensitivity can be specifically inactivated with ^{125}I-labeled antigen (1).

The specific adherence of sheep erythrocytes (SRBC) to lymphocytes to form rosettes is a useful technique to study the function of SRBC-specific T and bone marrow-derived (B) rosette-forming cells (RFC). T RFC bind fewer SRBC than B RFC (5–8) and tend to dissociate after resuspension of the pellet (9). If a metabolic inhibitor, sodium azide, is added immediately after resuspension of the pellet, viable T rosettes can be stabilized and purified for functional studies (10). Previous work has shown that small lymphocyte T RFC are neither helper cells in the antibody response nor effector cells in the DHR against SRBC (10). In the present investigation, effector cells in DHR and in vitro cytotoxicity correlated with non-RFC small T lymphocytes and with medium lymphoid T RFC.

Materials and Methods

Animals. Male C57BL/6 mice (Canadian Breeding Laboratories, Montreal, Quebec) 6–8 wk of age were used.

Medium. Tris (hydroxymethyl)-amino-methane-buffered (pH 7.4) Eagle's minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, N. Y.) was used for preparing cells.

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[1] *Abbreviations used in this paper*: DHR, delayed hypersensitivity response; DRBC, donkey red blood cells; GPS, guinea pig serum; KLH, keyhole limpet hemocyanin; MRBC, mouse red blood cells; PFC, plaque-forming cell; RFC, rosette-forming cell.

CMRL 1066 (GIBCO) supplemented with 10% fetal calf serum, glutamine, nonessential amino acids, and pyruvate was used for tissue culture. The pH was adjusted to 7.4 with a bicarbonate buffer. All cultures were incubated in a humidified incubator gased with 5% CO_2 in air.

Antigens and Immunization. Sheep or donkey blood was collected and stored in an equal volume of Alsever's solution. Allogeneic CBA mouse erythrocytes (MRBC) were obtained by bleeding from the neck into Alsever's solution immediately after cervical dislocation and were used on the same day.

Immunization was by the intradermal injection of 5×10^7 erythrocytes emulsified (1:1 by volume) in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). A vol of 0.05 ml was injected intradermally into the right foot pad of each mouse. In some experiments keyhole limpet hemocyanin (KLH) (Schwarz/Mann Div., Becton, Dickinson & Co., New York), emulsified with adjuvant, was used as antigen (1.25 μg KLH/mouse).

Sodium Azide and Neuraminidase Treatment. Sodium azide (Fisher Scientific Co., Pittsburgh, Pa.) and neuraminidase were used as previously described (10). Treatment with 0.05% azide, followed by washing in medium without azide, had no effect on the activity of effector cells.

Ammonium Chloride Treatment. In some experiments erythrocytes were removed from fractionated cell suspensions by NH_4Cl lysis (11) before testing for effector cell activity. Treatment with NH_4Cl had no effect on the activity of effector cells.

Preparation of Cell Suspension and Formation of Rosettes. Cells from spleen or axillary and popliteal lymph nodes were teased into MEM. Single cell suspensions were prepared and rosettes against SRBC were made as previously described (10).

Velocity Sedimentation. The technique of velocity sedimentation at unit gravity (12) was used to separate single cells and rosettes by differences in size (9, 10). Nucleated cells (8×10^7) plus 8×10^8 SRBC were loaded into each of two sedimentation chambers, and were sedimented for 1.5 h. Common fractions were pooled as indicated below. In some experiments, sodium azide was added to the rosette suspension and sedimentation medium to a final concentration of 0.05%.

Lymphocytes were categorized by diameter as defined by Metcalf and Wiadrowski (13). Small lymphocytes (<7 μm) sedimented at 3–4 mm/h, medium (7–11 μm) at 5–7 mm/h, and large (>11 μm) at greater than 8 mm/h (9).

Equilibrium Density Gradient Centrifugation. Equilibrium density gradient centrifugation (14) was used to separate cells by buoyant density. A continuous density gradient of bovine serum albumin ranging from 1.045–1.075 g/cm^3 was used. The gradient was spun at 4°C, at 3,800 *g* for 45 min. After centrifugation, 15 drop fractions were collected and the mean density of each fraction was determined by measuring the refractive index. Fractions were washed in tissue culture medium, counted, and tested for functional properties as described below.

Assay for Foot Pad Swelling. DHR was assayed by measuring the percent increase in foot pad volume after skin testing with antigen. Foot pad volume was proportional to the deflection observed on a Mettler 1600 balance (Fisher Scientific Co.) when the mouse foot was immersed in mercury to a mark made immediately distal to the lateral malleolus (15). Three readings were made on each foot and then averaged. The difference in readings before and after skin testing was proportional to the increase in foot pad volume. The percent increase in foot pad volume was calculated by dividing the difference by the original reading and multiplying by 100%.

Adoptive Transfer of DHR. A system was developed to transfer delayed hypersensitivity into normal syngeneic mice. Immune cells were intradermally injected with 5×10^7 SRBC (in 0.02 ml with a no. 27 needle) into the right foot pad of five mice per group. The other foot pad was injected with antigen alone as a control. The percent increase in foot pad volume was determined. In experiments involving cell separation, all the cells of each fraction were transferred with SRBC. The relative effector cell activity of immune cells was determined from the dose response graph in Fig. 2 and was expressed as the relative number of effector cells injected per mouse.

Assay for In Vitro Cytotoxicity. An in vitro microcytotoxicity assay similar to the method of Takasugi and Klein (16) was developed to test for cytotoxic cells directed against sheep fibroblasts which bear serologically detectable SRBC antigens (B. E. Elliott, unpublished results). Sheep fibroblasts were grown from a sterile biopsy of sheep skin (1 mm $\times$ 1 cm). After removing the outer layer, the skin was cut into small pieces with a scalpel blade and incubated in culture dishes (60 mm in diameter, no. 3002, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.), for 6–7 days. On the day of an experiment, the supernate was removed; the remaining monolayer of adherent fibroblasts was incubated at 37°C for 20 min with 0.025% trypsin in phosphate-buffered saline. Cells were then

washed in tissue culture medium, counted in 1% eosin yellow stain (Fisher Scientific Co.), and adjusted to 3×10^5 viable cells/ml. One drop (0.03 ml from a Pasteur pipette) of tissue culture medium and one drop of the fibroblast suspension were added to each well of a microtitration tray (Microtest Tissue II Culture Dishes, no. 3040, Falcon Plastics) to yield 100 viable fibroblasts per well. In some experiments, human melanoma tumor cells or rat fibroblasts were harvested from cultured cell lines and used as target cells.

Viable cells to be tested in the microcytotoxicity assay were adjusted to the required concentration and one drop of the cell suspension was added to each of five wells per group. Controls consisted of wells with fibroblasts alone.

Cytotoxicity was scored at 20 h or as indicated below. Two drops of 1% eosin in normal saline were added to each well. Nonadhering cells were removed by one gentle aspiration with a Pasteur pipette. Adhering viable fibroblasts (i.e., cells excluding the dye [17]) were counted with an Olympus inverted microscope (Olympus Corporation of America, New Hyde Park, N. Y.). The number of adhering target cells per control well varied between experiments, but was constant within an experiment.

The percent inhibition of fibroblasts was determined by the following formula: % Inhibition = $[1 - (x \div y)] \times 100\%$; where x is the average number of viable fibroblasts per well with lymph node cells, and y is the average number of viable fibroblasts in the control wells without lymph node cells. The relative effector cell activity after treatment of immune cells was the number of untreated cells per target cell (interpolated from the dose response curve in Fig. 3) required to yield the same percent fibroblast inhibition as treated cells.

In experiments involving velocity sedimentation, each cell fraction was cultured at a ratio of immune cells to target cells of 250:1, except for fractions in the high velocity regions (E, F, and G; Fig. 5) which were cultured at a ratio of 50:1. The enrichment of each fraction was calculated by dividing the relative immune cell activity of each fraction by the activity of an equivalent number of untreated cells. The relative effector cell number per fraction was the product of the enrichment and the total number of cells per fraction.

Trypsinization. Cells were washed in phosphate-buffered saline, and incubated with 0.025% trypsin (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline. After incubation at 37°C, the cells were washed twice in MEM.

Treatment with Antisera. In all experiments a two-step procedure was used. Antiserum at the required dilution in phosphate-buffered saline was added to the pellet of cells to yield a final cell concentration of 5×10^6/ml. Cells were incubated at 25°C. After 30 min complement (C) was added to a final dilution of 1/8. The cells were then incubated at 37°C for 45 min. Controls consisted of cells incubated alone, or with antiserum (1/0), or with C. After the final incubation, cells were washed in 1066; viable cells were counted with 1% eosin stain and functional tests were performed, as described below.

AKR anti-C3Hθ serum (a gift from Dr. R. S. Kerbel, Queens University, Kingston, Ontario) and goat antimouse immunoglobulin (anti-Ig) serum (Melloy) were used with guinea pig serum (GPS) at a final dilution of 1/8. Anti-Ly-4.2 serum (a gift from I. McKenzie, University of Melbourne, Melbourne, Australia) was used with normal rabbit serum (1/8) as a source of C. This alloantiserum is specific for B cells (I. McKenzie, personal communication). Rabbit serum was absorbed with C57BL/6 thymus and spleen cells (1 volume of cells to 7 volumes of serum).

Assay for Cross-Reactivity of Antibody. Anti-SRBC antibody was obtained from hyperimmune mice and the hemagglutination titer (18) against SRBC, donkey erythrocytes (DRBC), or MRBC was determined. Cross-reactivity with SRBC was calculated by dividing the titer of each erythrocyte with that of SRBC, and multiplying by 100%.

Assay for Cross-Reactivity in the Production of Plaque-Forming Cells (PFC). SRBC-immune lymph node cells were cultured with 1×10^7 SRBC or DRBC were assayed 4 days later by the technique of Cunningham and Szenberg (20). Cross-reactivity was determined by dividing the number of anti-SRBC PFC in cultures with DRBC, by the number of anti-SRBC PFC in cultures with SRBC, and multiplying by 100%. Since there is very little cross-reactivity at the antibody level with these antigens (Table I), the majority of cross-reactivity observed is thought to be at the level of helper T cells (21).

Results

Kinetics of Induction of DHR. Mice were immunized with SRBC, with or without adjuvant, in one foot pad. At various times, the other foot pad was skin

tested, and the percent increase in foot pad volume was measured 20 h later. The maximum foot pad swelling occurred when immune mice were challenged 9 days after immunization (Fig. 1). Immunization with a low antigen dose, or without adjuvant, resulted in a much smaller reaction. Both mononuclear and polynuclear cells infiltrated into the foot pad of immune animals after skin testing with antigen as reported by other investigators (1). Normal animals skin tested with SRBC gave no reaction. The subsequent experiments were designed to characterize the DHR and cytotoxicity reaction which were maximal at 9 days after immunization.

Dose Response Relationship. The effect of the dose of immune cells on effector cell activity was determined for both DHR and in vitro cytotoxicity.

For DHR, doses of 5×10^4–1×10^8 SRBC-immune lymph node cells were transferred intradermally with antigen: the percent increase in foot pad volume (measured after 18 h) was proportional to the immune cell dose. SRBC alone, immune cells alone, or nonimmune cells plus SRBC produced no swelling (Fig. 2).

For cytotoxicity, immune cells were cultured at ratios of lymph node cells to target cells ranging from 50:1 to 1,000:1. Cytotoxicity against sheep fibroblasts was directly proportional to the number of immune cells at ratios of immune to target cells between 50:1 and 800:1 (Fig. 3). No cytotoxicity was observed when normal lymph node cells were cultured with sheep fibroblasts, or when SRBC-immune lymph node cells were cultured with human melanoma cells or with rat fibroblasts (unpublished result). These data suggest that the cytotoxic cells act specifically against sheep fibroblasts, and that background target cell killing is minimal.

Effect of Antisera on Effector Cell Activity. SRBC-immune lymph node cells (2.5×10^7/group) were treated with different dilutions of anti-θ serum or anti-Ig serum, plus C, and were tested for DHR or in vitro cytotoxicity. Foot pad

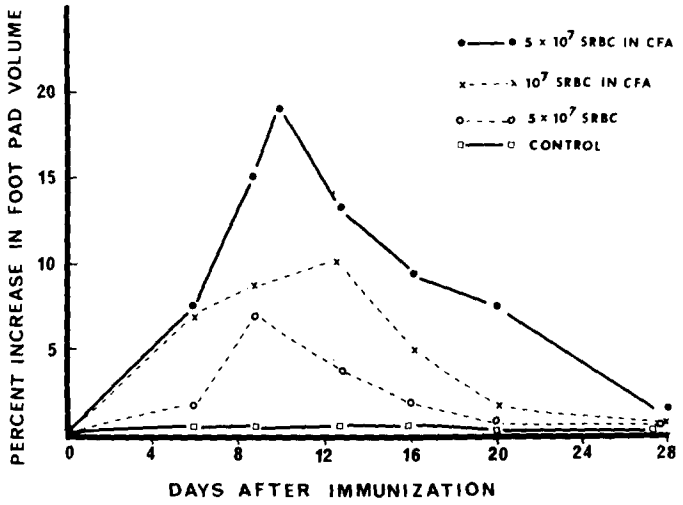

FIG. 1. Kinetics of induction of DHR. Mice immunized with 5×10^7 SRBC in CFA, 1×10^7 SRBC in CFA, or 5×10^7 SRBC alone, were challenged with 5×10^7 SRBC at various times after immunization. Foot pad volumes were measured 18 h after challenge. A control consisted of untreated animals.

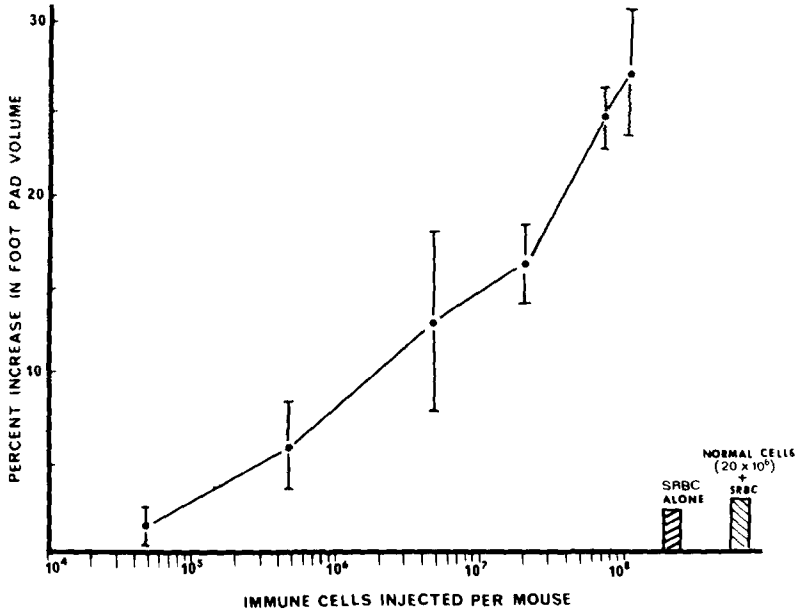

FIG. 2. Effect of immune cell dose on foot pad swelling. Doses of immune lymph node cells from 9-day immune mice were injected intradermally with 5×10^7 SRBC into one foot pad. Controls consisted of the other foot pad injected with SRBC alone. Each point represents the mean increase in foot pad volume (after 18 h) of five animals ± the standard error.

volumes were measured 18 h after foot pad challenge and microcytotoxicity was scored after 36 h of culture with target cells.

Greater than 90% of the effector cell activity was lost after treatment with anti-θ serum (1/10) plus GPS (Table I). No activity was lost after incubation with GPS or anti-θ serum alone. Similar results were obtained with two anti-θ sera, supplied by I. McKenzie and R. S. Kerbel. In contrast, treatment with anti-Ig serum (dilution 1/8) plus C or with anti-Ly-4.2 serum plus rabbit C, did not inhibit effector cell activity, although specific killing of immune lymph node cells was observed (Table I).

Size and Rosette-Forming Ability of Effector Cells. Without prior rosette formation, DHR effector cells (assayed 18 h after foot pad challenge) sedimented as small and medium lymphocytes (fractions A, B, and C; Fig. 4 B).

When immune cells were subjected to rosette formation before sedimenting with azide, both T- and B-type rosettes sedimented at velocities greater than 6.5 mm/h (Fig. 4 A). Fractions E and F contained medium lymphocyte T-type rosettes. Fraction G contained multilayered rosettes, previously shown to be antibody secreting cells (9). Significant DHR activity remained in the nonrosette small lymphocyte fraction; but the medium lymphocyte effector cells sedimented as rosettes into fractions E and F. No DHR activity was associated with fraction G. Without azide to stabilize T rosettes, the medium lymphocyte rosettes dissociated and all effector cells sedimented as single cells in the small and medium lymphocyte regions (Fig. 4 B).

Cytotoxic effector cells displayed similar sedimentation properties. When the

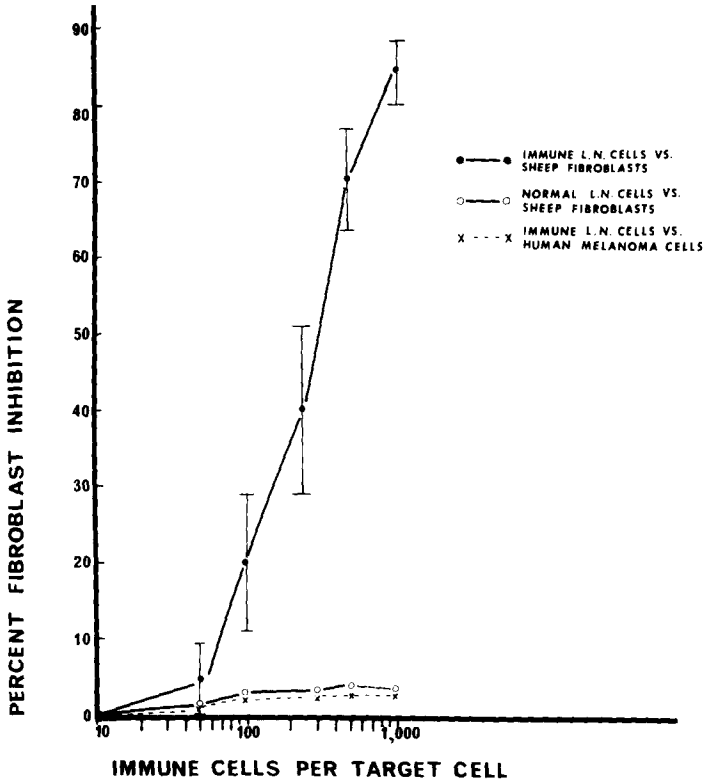

FIG. 3. Effect of immune cell dose on in vitro cytotoxicity. Lymph node cells from 9-day immune animals were cultured at different immune cell to target cell ratios in the wells of a microtitration tray. Controls consisted of immune cells cultured with human melanoma tumor cells and normal lymph node cells cultured with sheep fibroblasts. Each point represents the mean percent fibroblast inhibition (after 20 h) ± the standard error.

percent fibroblast inhibition was scored at 20 h, cytotoxicity was associated only with medium lymphocytes (fractions B and C; Fig. 5 A); after rosette formation these cells sedimented as rosettes in fractions E and F. At 36 h, cytotoxicity was associated with both nonrosette-forming small lymphocytes (fraction A) and medium lymphocyte rosettes (fractions E and F) (Fig. 5 B). Without azide to stabilize T rosettes, effector cells sedimented as single cells (Fig. 5 A).

Anti-θ Treatment of DHR Effector Cells. After treatment with anti-θ serum plus C, 90% of the effector cell activity of both the nonrosette-forming small lymphocytes (fraction A) and the medium lymphocyte RFC (fraction EF) was lost. Incubation alone, or with sera alone, had no effect on effector cell activity (Fig. 6).

Kinetics of Fractionated Effector Cells. The kinetics of effector cell activity in DHR and cytotoxicity were compared. Rosettes were made from immune lymph node cells, stabilized by sodium azide, and separated by velocity sedimentation. Activity of fractionated effector cells in DHR and in vitro cytotoxicity was measured at various times after challenge with antigen.

Medium lymphocyte effector RFC produced a maximum foot pad swelling at

TABLE I

Effect of Antisera Treatment on DHR and Cytotoxicity

DHR			Cytotoxicity	
Treatment	Recovered cells	Survival of activity	Treatment	Survival of activity
	%	%		%
Control (37°C)	100	100	Control (37°C)	100
c′ *	91	80 ± 10	c′ *	83 ± 12
θ‡ (1:20)	90	100 ± 8	θ‡ (1:10)	78 ± 8
c′ + θ (1:20)	60	12 ± 2	c′ + θ (1:10)	9.5 ± 5
c′ + θ (1:60)	56	28 ± 12	c′ + θ (1:30)	15 ± 5
c′ + θ (1:180)	76	68 ± 14	c′ + θ (1:90)	60 ± 20
c′ + θ (1:540)	84	100 ± 10	c′ + θ (1:270)	83 ± 13
Anti-Ig (1:8)	100	84 ± 13	anti-Ig	95 ± 14
c′ * + anti-Ig (1:8)	44	96 ± 11	c′ * + anti-Ig	87 ± 16
anti-Ly-4.2§ (1:10)	96	NT	anti-Ly 4.2	80 ± 11
c′ ‖ + anti Ly-4.2 (1:10)	48	NT	c′ ‖ + anti-Ly-4.2	95 ± 12

* c′, guinea pig C.
‡ θ, AKR anti-$C_3H\theta$.
§ (BALB/c × SWR)F_1 anti-B10.D2 (a gift from I. McKenzie).
‖ c′, rabbit C.

12 h; the maximum swelling produced by the nonrosette-forming small lymphocytes occurred at 20 h. Fractions C and D were completely unresponsive. An equivalent number of unfractionated cells produced a foot pad swelling response which was a composite of the response of both the small and medium lymphocyte effector cells (Fig. 7 A). No swelling was observed in the first 4 h. However, when anti-SRBC serum plus SRBC were injected intradermally there was an immediate swelling at 4 h which disappeared after 10 h; this immediate reaction was distinct from the delayed cell-mediated footpad swelling reaction (B. E. Elliott, unpublished result).

The medium lymphocyte effector RFC (fractions E and F) developed detectable cytotoxic activity after 15 h of culture with fibroblasts. Nonrosette-forming small lymphocytes (fraction A) were inactive at 22 h; they developed detectable cytotoxic activity after 36 h of culture. Fractions C and G produced no activity at any time (Fig. 7 B).

Comparison of the Buoyant Density of Medium Lymphocyte Effector Cells in DHR and In Vitro Cytotoxicity. For further comparison of DHR and cytotoxic effector cells, medium lymphocytes (fraction B and C) were separated from immune lymph node cells by velocity sedimentation and subjected to equilibrium density gradient centrifugation. Nucleated cells were present in both the light and dense regions of the gradient and were pooled into six density fractions (Fig. 8). The majority of cells in fractions 1, 2, and 3 were medium lymphocytes (less dense); and the majority of cells in fractions 5 and 6 were small lymphocytes (more dense) (14). The latter represent contamination from fraction A of the sedimentation. The relative DHR and cytotoxic effector cell numbers per fraction were determined.

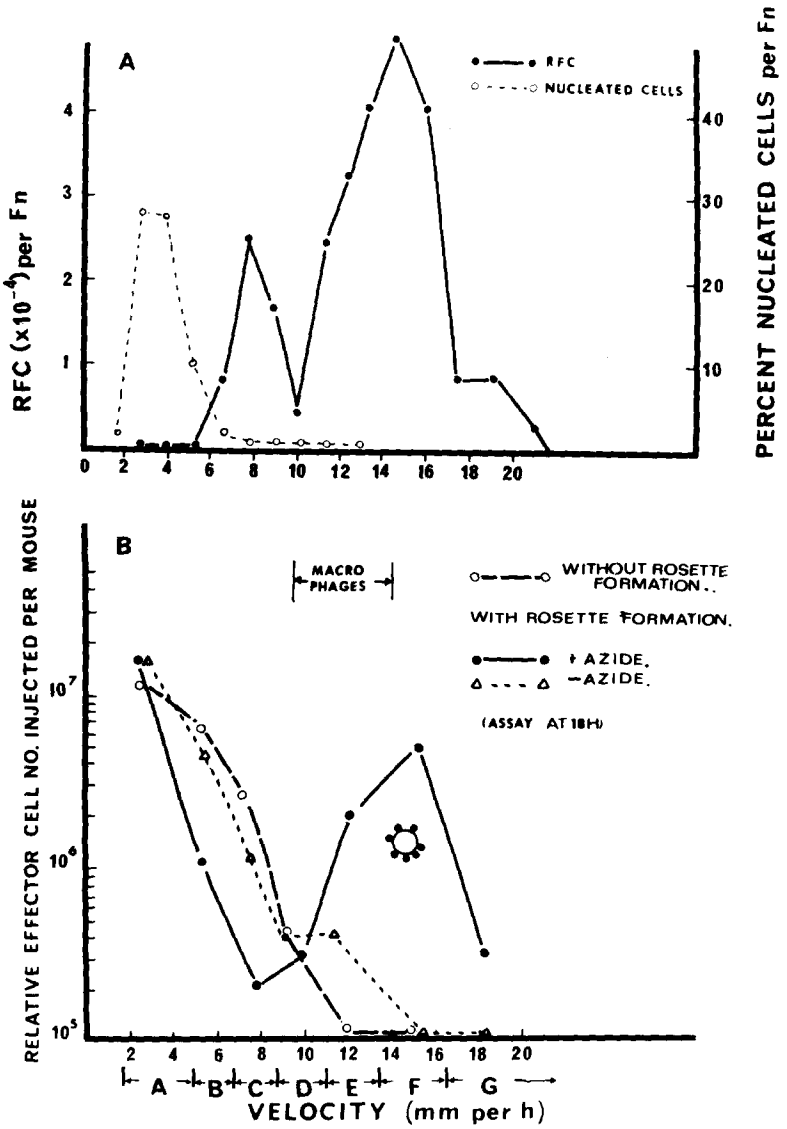

FIG. 4. Effect of velocity sedimentation on DHR effector cells. (A) Rosettes were made from immune lymph node cells and subjected to velocity sedimentation with sodium azide present. The absolute number of rosettes per fraction and the percent of total nucleated cells per fraction is shown. (B) Immune cells were subjected to velocity sedimentation (*a*) without prior rosette formation, or (*b*) with rosette formation plus sodium azide, or (*c*) with rosette formation but without sodium azide. Each fraction was assayed for DHR and the relative effector cell number injected per mouse was determined from Fig. 2. Each point was normalized to represent equivalent units of sedimentation velocity.

Effector cells in both delayed hypersensitivity and in vitro cytotoxicity were associated with fractions 2 and 3 (1.0565–1.0640 g/cm^3). A minor peak of DHR activity in fraction 6 (>1.070 g/cm^3) correlated with the contaminating small lymphocytes present in that fraction.

Cross-Reactivity of Effector Cells. Cross-reactivity in the induction of

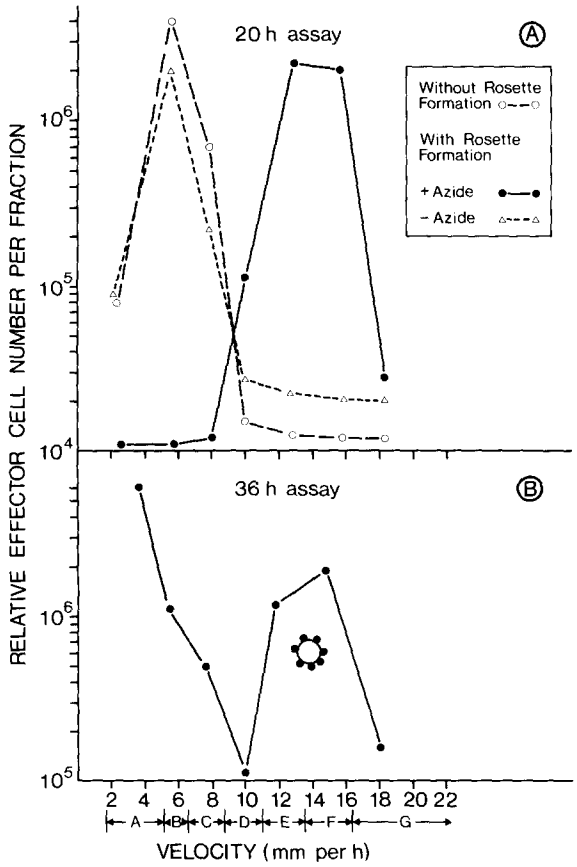

FIG. 5. Effect of velocity sedimentation on cytotoxic effector cell activity. Immune lymph node cells were sedimented as in Fig. 4 B. Each fraction was cultured in a microcytotoxicity test with sheep fibroblasts. (A) The percent fibroblast inhibition was scored after 20 h. Fractions B and C (without rosette formation) produced 70 and 73% inhibition, respectively. Fractions E and F (with rosette formation plus azide) produced 78 and 92% inhibition, respectively. The relative number of effector cells per fraction was determined. The distributions are normalized to an arbitrary scale. (B) The percent fibroblast inhibition was scored after 36 h.

effector cells was determined by testing lymph node cells immunized against DRBC, SRBC, MRBC, or KLH, for DHR or cytotoxicity against SRBC or sheep fibroblasts. Foot pad volumes were measured 18 h after challenge; fibroblast inhibition was scored after 36 h. Cross-reactivity was calculated by dividing the relative activity of effector cells immunized with KLH, DRBC, or MRBC, by the relative activity of the SRBC immune effector cells and multiplying by 100%. The results in Table III indicate significant cross-reactivity with DRBC, of both DHR and cytotoxic effector cells; but no cross-reactivity with MRBC or KLH.

The degree of cross-reactivity of these antigens with SRBC in the induction of effector cells is similar to that observed in helper cell activity in the production of PFC. Antibody against SRBC cross-reacted much less with DRBC and MRBC (as determined by a hemagglutination test) (Table II).

The specificity of erythrocyte binding by effector cells was determined by subjecting SRBC-immune lymph node cells to rosette formation with MRBC

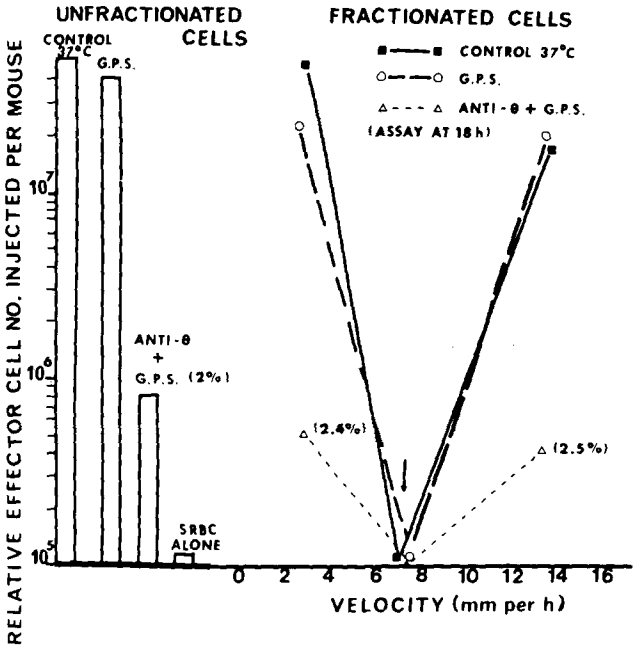

FIG. 6 Effect of anti-θ serum on fractionated effector cells in DHR. Immune cell fractions from a velocity sedimentation (plus azide) with prior rosette formation were incubated alone, or with C, or with anti-θ serum plus C. Each group was tested for DHR (in an 18 h assay) and the relative number of effector cells was determined.

before sedimenting. Anti-SRBC effector cell activity remained in the small and medium lymphocyte regions; medium lymphocyte effector RFC did not bind MRBC. If rosettes from SRBC-immune cells were made against DRBC before sedimenting, significant effector cell activity was associated with the medium lymphocyte rosette fraction. This activity represented 25% cross-reactivity with DRBC of SRBC-immune medium lymphocyte effector cells (unpublished observations).

Effect of Trypsinization on the Function of Effector Cells. The nature of antigen receptors on rosette-forming effector cells was investigated by treatment of immune cells with trypsin. After 5 min of trypsinization, less than 10% of the original number of RFC still formed rosettes (Table III). These results suggest that antigen receptors were altered or removed by enzyme treatment. No loss of DHR or cytotoxic effector cell activity was observed after periods of trypsinization ranging from 10 to 40 min. When cells were washed after 10 min of trypsinization, regeneration of rosette-forming ability was observed within 60 min; by 180 min, 95% of the original number of T RFC and B RFC had regained the ability to bind SRBC (Table IV).

Discussion

Since both T and non-T cells may be involved in cellular immune reactions (22), it is important to define clearly the type of effector cell detected. In the present work, 90% of the activity in DHR and cytotoxicity was abolished by anti-θ serum

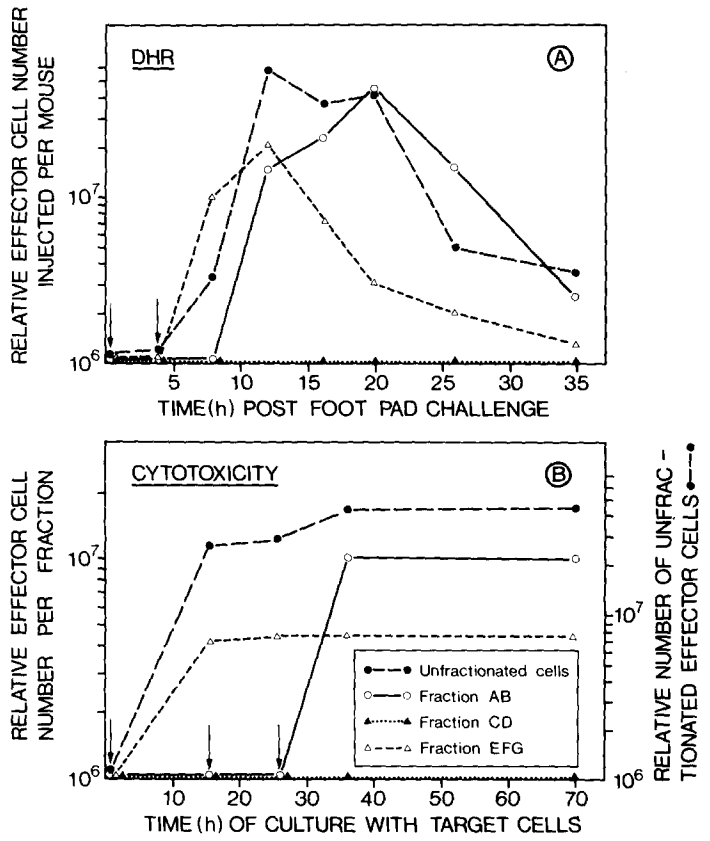

FIG. 7. Kinetics of fractionated effector cells. (A) Fractions were pooled into AB(2–6.5 mm/h), CD (6.5–11 mm/h), and EFG(>11 mm/h). Each fraction was tested for DHR activity and the relative effector cell number injected per mouse was determined at various times after culture. (B) Immune lymph node cells were subjected to rosette formation and sedimented with azide present. Fractionated cells were cultured with sheep fibroblasts, and the percent fibroblast inhibition was determined at various times after culture. The relative effector cell number of fractions (A + B), (C + D), and (E + F) was plotted vs. time.

plus C, but was insensitive to anti-Ig serum or anti-Ly-4.2 serum plus C. T cells are therefore the main mediators of DHR and cytotoxicity observed; Ig-bearing B cells were not involved.

The response was antibody independent, since multilayered rosettes secreting 19S and 7S antibody, which sedimented in fraction G (B. E. Elliott, unpublished result), were inactive in both in vivo and in vitro assays. Furthermore, the kinetics of the foot pad swelling response after the transfer of immune cells into normal animals was characteristic of a delayed response in contrast to the 4 h antibody-mediated response which disappeared after 10 h (Fig. 7 A). The DHR response was thus distinct from the immediate Arthus-type reaction (23).

Activity in DHR and in vitro cytotoxicity was associated with a nonrosette-forming small lymphocyte and a medium lymphoid T RFC. Activity of both cell types was sensitive to anti-θ serum plus C. The antigen-binding property of the medium lymphocyte effector cells was characteristic of T RFC. The effector cell rosette dissociated in suspensions without azide (Figs. 4 and 5).

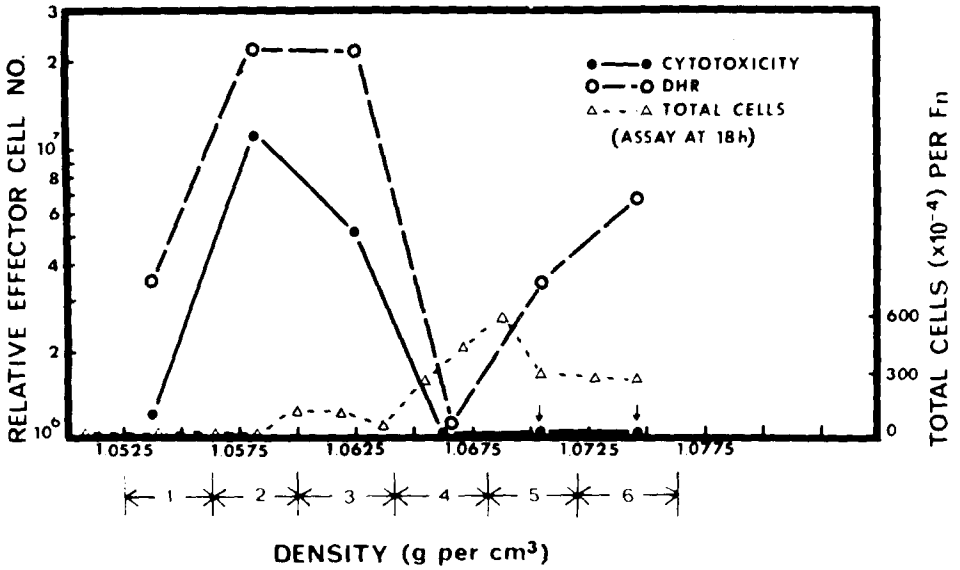

FIG. 8. Comparison of buoyant density of medium lymphocyte effector cells in DHR and in vitro cytotoxicity. Medium lymphocytes, separated by velocity sedimentation (fractions B and C), were subjected to equilibrium density gradient centrifugation. Density regions were pooled into six fractions and each fraction was assayed for delayed hypersensitivity or in vitro cytotoxicity (the immune cell to target cell ratio was 250:1). Foot pad swelling (16% in fractions 2 and 3) and percent fibroblast inhibition (76% in fraction 2) were assayed after 18 h, and the relative number of effector cells per fraction was determined.

TABLE II

Comparison of Cross-Reactivity of T- and B-Cell Functions

Function	Cross-reactivity with SRBC		
	DRBC	MRBC	KLH
	%	%	%
T			
Induction of cytotoxic cells	55 ± 8*	0	0
Effector cells in DHR	28 ± 4	0	0
Helper activity in PFC production	40 ± 5	NT	NT
B			
Anti-SRBC antibody	8	1	NT

* The mean ± the standard error of at least two experiments is indicated.

The medium lymphocyte effector RFC sedimented as rosettes are a mean velocity of 13–15 mm/h (Figs. 4 and 5). The number of bound SRBC required to make a medium lymphocyte sediment at this velocity is 7–10. This velocity has previously been associated with small lymphocyte B rosettes binding an average of 18 SRBC per RFC (9). However, the medium lymphocyte was larger, and sedimented at 5–7 mm/h compared to the small lymphocyte which sedimented at 3–4 mm/h (24). Assuming the following mean volumes, SRBC, 32 μm^3 (25); small

TABLE III
Effect of Trypsinization on Rosette Formation and Activity Effector Cell

Incubation time	Treatment (0.025% trypsin)	Survival of activity		
		RFC*	DHR ‡	Cytotoxicity*
min		%	%	%
10	+	12 ± 10	90 ± 16	130 ± 18
20	+	37 ± 12	95 ± 20	115 ± 12
40	+	35 ± 14	99 ± 15	130 ± 18
40	–	95 ± 10	95 ± 10	100 ± 20
0	–	100	100	100

* The mean ± the standard error of two experiments is indicated.
‡ The mean ± the standard error of five animals per point is indicated.

TABLE IV
Recovery of T and B RFC after 10 min Trypsinization

Incubation time	Recovery of RFC	RFC class	
		T	B
min	%	%	%
Treated			
0	18 ± 6		
5	22 ± 8		
15	24 ± 8		
30	13 ± 10		
60	75 ± 15		
120	80 ± 13		
180	95 ± 18	63	32
Untreated			
0	100	60	40
180	95 ± 20	57	38

lymphocyte, 172 μm^3 (14); medium lymphocyte, 400 μm^3 (14); the following volumes can be calculated, small lymphocyte with 18 SRBC bound, 750 μm^3 and medium lymphocyte with 10 SRBC bound, 720 μm^3. Since the medium lymphoid T rosette with 10 SRBC bound was the same size as the small lymphoid B rosette (with 18 SRBC), both types of rosettes sedimented at the same velocity (13–15 mm/h). The effector RFC observed therefore, bound an average of 7–10 SRBC, were sensitive to anti-θ serum plus C, and were therefore, T RFC.

The present work is a direct demonstration of a function of medium lymphocyte T RFC in cell-mediated immunity against SRBC. These results imply that the medium lymphocyte effector T cells bind SRBC specifically. The degree of cross-reactivity, between DRBC and SRBC, in the induction of effector cells in DHR and cytotoxicity was greater than that of anti-SRBC antibody

(Table II). Similarly, Cooper and Ada (1) reported high cross-reactivity between different Salmonella flagellins of effector cells in DHR, but only slight cross-reactivity of antisera directed against the same flagellin antigens. Similar cross-reactivity, between DRBC and SRBC, of SRBC-immune cells in the production of PFC was observed (Table II). However, cross-reactivity in the production of PFC may represent a nonspecific stimulatory T-cell function (26) or a macrophage function (27). Since the degree of cross-reactivity is an indication of specificity, these results suggest that the specificity of effector T cells in DHR is similar to those in in vitro cytotoxicity; but both are different from that of B cells.

Although trypsinization inhibited the rosette-forming ability of immune lymph node cells, these cells were capable of regenerating the ability to form rosettes. The function of effector cells in in vitro cytotoxicity and DHR was uninhibited (Tables III and IV). These results suggest that receptors for antigen on medium lymphocyte effector cells are endogenous, although antibody from contaminating B cells may have been passively absorbed.

The small lymphocyte required a longer period than the medium lymphocyte to produce maximum swelling in vivo and detectable cytotoxicity in vitro (Fig. 7). The small lymphocyte effector cell may transform into a medium lymphocyte effector cell before producing its maximum effect. Such a transformation from small to medium lymphocytes has been demonstrated in the graft-vs.-host reaction in vivo (28) and in the development of hypersensitive lymphocytes in vitro (29).

However, small lymphocyte nonrosette-forming cells may become active without transformation to medium lymphocytes. Evans et al. (30) have shown that specific T cells activate macrophages with a specific macrophage-arming factor in a syngeneic or allogeneic (tumor) immune system. Macrophages, activated by small T lymphocytes, could conceivably be killer cells in the present system. Mononuclear cells (of host origin [23]) which infiltrate into the skin reactive site in vivo may become armed by the immune cells present and may enhance the immune reaction against injected antigen. This would provide a plausible explanation for the delayed effect shown with the nonrosette-forming small T-lymphocyte population. Thus, although T cells are necessary for cytotoxicity and DHR in the present system (Table I), cells other than T cells may also be involved.

Sensitivity to anti-θ serum, density, size, antigen-binding properties, and kinetics failed to distinguish between effector cells in DHR and in vitro cytotoxicity. The same effector cell may be detected in both assays. Factors which produce positive skin reactions in DHA (31), may be secreted by the same cell type that mediates cytotoxicity in vitro. DHR may therefore, be an in vivo manifestation of the same type of cell-mediated immunity detected by in vitro cytotoxicity.

Summary

Effector cells in delayed hypersensitivity and in vitro cytotoxicity were studied in lymph node cells from animals immunized with sheep erythrocytes (SRBC) in complete Freund's adjuvant. Delayed hypersensitivity response (DHR) was assayed by the increase in foot pad swelling after the intrafoot pad injection of

immune cells plus antigen. Cell-mediated cytotoxicity against SRBC was assayed by a microcytotoxicity test with sheep fibroblasts as target cells. Effector cells were antigen specific, sensitive to anti-θ serum plus complement (C), and insensitive to anti-Ig serum plus C. A nonrosette-forming (non-RFC) small lymphocyte effector T cell and a rosette-forming medium lymphocyte effector T cell were isolated by velocity sedimentation. The small lymphocyte non-RFC required a longer time than the medium lymphocyte RFC effector cell to produce maximum activity. Buoyant density failed to distinguish medium lymphocyte effector cells in DHR and in vitro cytotoxicity.

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