Brief Definitive Report

ANTIGEN-BINDING T CELLS AS HELPER CELLS

Separation of Helper Cells by Immune Rosette Formation*

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Helper cells active in cooperative responses are specific to the inducing antigen (1). They are also susceptible to suicide with radioiodinated antigen (2, 3). This suggests that helper cells may express antigen-specific receptors. However, efforts to enrich or to purify helper cells on the basis of antigen-binding capacity either by the use of antigen-coated columns (4) or by rosetting techniques (5–7) have so far been unsuccessful. We here report that helper cells can be enriched from the spleen T cells of immunized mice by using their capacity to bind sheep erythrocytes coated with immunogen.

Materials and Methods

Animals. CBA mice bred at the Dept. of Serology and Bacteriology were used throughout. The mice were subjected to the experiments at the age of 3–6 mo.

Antigens. The preparation of chicken gamma globulin (CGG) and dinitrophenylated (DNP-)CGG has been described previously (8). Donkey red blood cells (DRC) were kindly donated by Dr. J. Ivanyi. They were trinitrophenylated (TNP-DRC) as described by Rittenberg and Pratt (9) using 20 mg of TNP sulfonic acid per 1 ml of packed donkey red cells for coupling.

Immunizations. Mice were injected intraperitoneally (i.p.) with 200 μg of alum-precipitated CGG together with 10^9 *Hemophilus pertussis* bacteria (8). The spleens of these mice were used as a CGG helper cell source 6 days after antigen injection. Other mice were injected i.p. with 0.2 ml of 10% DRC and their spleen cells used 6 days later as a source of DRC helper cells. Spleen cells from mice injected three times i.p. with TNP keyhole limpet hemocyanin (TNP-KLH) absorbed on bentonite (9) were used as a source of TNP-immune cells. Normal spleen cells from 3- to 4-mo old mice were also used as a source of antibody-forming cell precursors.

Preparation of Cell Suspensions. Spleens were teased into suspension as described earlier (10), and the erythrocytes lysed with a 0.83% aqueous solution of NH_4Cl. The viable nucleated cells were counted by the trypan blue exclusion method.

Fractionation of Cells. Spleen cells from CGG or DRC immune mice (helper cells) were enriched for T cells by passage through Fenwall-Leukopak nylon wool columns as described (11). 5–7% of the passed cells stained with fluorescein-conjugated sheep antimouse immunoglobulin.[1] The nylon wool-passed T-enriched cells were used for helper cell rosetting. 95–100% of the nylon wool column-passed cells were susceptible to lysis with anti-T serum (sheep anti-DBA/2 mouse brain) plus complement.

The anti-T serum lysed 100% of cortisone-resistant thymocytes at a dilution of $^1/_{64}$ and 40–50% of spleen cells at a dilution of $^1/_{16}$ to $^1/_{32}$. Adsorption of the anti-T serum with DBA/2 brain abolished its lytic activity against mouse spleen cells.

Rosette Formation and Isolation of Rosette-Forming Cells. Nylon wool column-purified spleen

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[1] Kindly donated by Professor Astrid Fagraeus, Stockholm.

cells from CGG-immunized mice were mixed with CGG-coated (subagglutinating doses of chicken anti-SRBC) sheep red blood cells (SRBC) in proportions of 1:50. The mixture was incubated in RPMI 1640 medium containing 3% FCS at 37°C for 10 min, centrifuged at 200 *g* for 5 min at +4°C, kept at room temperature for 15 min, and in an ice bath for an additional 30 min. Thereafter the pellet was disrupted by gentle shaking, the suspension diluted with cold Dulbecco's salt solution supplemented with 3% FCS to give a final concentration of 5×10^6 nucleated cells/ml. 20 ml of the suspension containing cells forming rosettes with CGG-coated sheep red blood cells (CGG-RFC), free lymphocytes, and erythrocytes was fractionated by 1-*g* velocity sedimentation as described by Miller and Phillips (12). The fractions containing only RFC and only non-RFC were separately pooled and the SRBC lysed with NH_4Cl. The RFC and non-RFC were then tested for helper activity as described below.

Spleen T cells from DRC-immunized mice forming rosettes with DRC were fractionated and tested in a similar way.

Tissue Culture Conditions. Marbrook type cultures were used for cooperative responses in vitro as described (10). The numbers of helper cells needed for optimal antibody responses under these conditions are ca. 10^5–10^6 per culture (10). Spleen cells, nylon wool-filtrated spleen cells, RFC, or non-RFC were mixed with 15×10^6 TNP-KLH immune or normal spleen cells, and the mixture was challenged with 2 μg of DNP-CGG/ml or with 1×10^6 TNP-DRC/ml. The culture medium was MEM + 5% calf serum. After 4 days in culture, the cells were harvested, and the numbers of anti-DNP antibody-forming cells (anti-DNP-AFC) per culture assayed (10). Only direct (IgM) AFC were tested. In the tables means ± SE are given.

Results

Proportions of T Cells Forming Antigen-Specific Rosettes. Out of nylon wool-filtered CGG-immunized spleen cells 0.5–1% formed rosettes with CGG coated SRBC. The same percentages of DRC-RFC were found in nylon wool-filtrated DRC-immune spleen. The isolated RFC were sensitive to anti-T serum plus complement. Less than 4% of CGG-RFC had detectable surface immunoglobulin, while less than 7% of non-CGG-REF had detectable surface immunoglobulin.

Enrichment of Helper Cells in Rosette-Forming Cell Fraction. Depletion of B cells by the use of nylon wool filtration improved the efficiency of CGG-primed spleen cells as helper cells (Table I). Treatment of spleen cells with anti-T serum and complement reduced the number of anti-DNP-AFC to background levels (190 AFC/culture). However, a 100-fold enrichment in helping efficiency was seen in the fraction of lymphocytes forming rosettes with CGG-coated sheep cells, e.g. 10^4 CGG-RFC gave 713 AFC per culture as compared to 790 by 10^6 nylon wool column-passed cells (Table I). A decrease in helping activity was seen in non-RFC fractions as compared to the unfractionated cell population. With higher number of non-RFC a weak helper activity could be detected in some experiments. As shown in Table I, 10^7 non-RFC gave 503 AFC as compared to 790 by 10^6 cells of the unfractionated population. These findings indicate that there is a selective recovery of helper cells in the CGG-RFC population.

The Specificity of Rosette-Forming Helper Cells. To test the specificity of helper RFC, nylon wool column-passed spleen cells from CGG-immunized and from DRC-immunized mice were mixed in equal proportions. The cells forming rosettes with DRC were then isolated from the mixture by velocity sedimentation. The helper activity in the DRC-RFC fraction, as well as that in the fraction depleted of DRC-RFC, was then compared to that in the original mixture. Cells in the DRC-RFC fraction showed an enrichment (ca. 1.5 times) in helper activity

TABLE I

The Effect of Various Procedures on the Helping Efficiency of CGG-Primed Spleen Cells

No. of helper cells added*	Challenge	Anti-DNP-AFC/culture ± SE				
		Treatment of helper cells:				
		Nil	Nylon wool filtration	anti-T + C′	CGG-RFC	Non-CGG-RFC
10^4	DNP-CGG‡	ND	ND	ND	*713 ± 77*	280 ± 78
10^5	"	ND	ND	ND	*870 ± 257*	167 ± 35
3×10^5	"	303 ± 143	103 ± 22	110 ± 71	ND	73 ± 37
10^6	"	413 ± 135	*790 ± 119*	180 ± 61	1,073 ± 152	ND
3×10^6	"	150 ± 67	470 ± 61	126 ± 62	ND	ND
10^7	"	ND	ND	ND	ND	506 ± 256

Values higher than background ($P < 0.05$) are in italics.

* Given numbers of helper cells were added to 15×10^6 spleen cells from TNP-KLH immune mice. The spleen cells alone (without the addition of helper cells) gave a response of 190 ± 79 anti-DNP-AFC/culture when challenged with DNP-CGG.

‡ 2 μg of DNP-CGG/ml.

TABLE II

Specificity of Immune Rosette-Forming Helper Cells

Fractionation of helper cells*	Challenge‡	Anti-DNP AFC/culture ± SE		
		No. of helper cells added:		
		10^4	10^5	10^6
Nil	DNP-CGG	227 ± 136	*397 ± 79*	*530 ± 111*
	TNP-DRC	693 ± 289	*740 ± 164*	367 ± 192
Cells forming rosettes with DRC	DNP-CGG	13 ± 13	3 ± 3	27 ± 18
	TNP-DRC	*1,050 ± 40*	*517 ± 116*	*420 ± 67*
Cells not forming rosettes with DRC	DNP-CGG	173 ± 126	ND	*527 ± 75*
	TNP-DRC	157 ± 137	170 ± 170	327 ± 78

* Spleen cells from CGG immune mice or from DRC-immune mice mixed in equal proportion after filtration through Fenwall Leukopak nylon wool.

‡ The given number of helper cells were added to 15×10^6 normal spleen cells and the mixture was challenged either with 2.0 μg/ml DNP-CGG or 1×10^6 TNP-DRC. The normal spleen cells alone gave a response of 93 ± 48 anti-DNP-AFC/culture when challenged with DNP-CGG and 137 ± 58/culture when challenged with TNP-DRC. Values higher than background ($P < 0.05$) in italics.

when challenged with TNP-DRC, but no helper activity when challenged with DNP-CGG (Table II). Cultures with helper cells from the non-DRC-RFC fraction displayed unaltered responses to DNP-CGG as compared to the unfractionated mixture, but gave only a weak response when challenged with TNP-DRC. With high helper cell doses, however, this decreased response to TNP-DRC challenge was overcome (Table II).

Discussion

Our results show that helper T cells can be enriched with the use of rosetting techniques. The enrichment is specific, e.g. from a mixture of helper cells of two different specificities a given specificity can be selectively isolated. The T-cell nature of the helper cells is shown by their sensitivity to anti-T serum and complement, and by the fact that the majority of the B cells were removed by filtration through the nylon wool column.

Although T cells binding a variety of antigens like sheep red blood cells, synthetic polypeptides, and keyhole limpet hemocyanin (13–15) have been demonstrated, the efforts to recover helper cells by specific rosetting have so far been unsuccessful (5–7). The discrepancies between previous reports and this work may perhaps be explained by a number of differences. We have used purified immune T cells as a starting cell population. Also, as T cells generally show a relatively weak binding of antigen (13, 15), we expected helper RFC to be fragile. The 1-*g* velocity sedimentation technique allows a gentle separation of RFC, as the rosettes are not exposed to strong *g* forces as in various centrifugation techniques. Also, by the use of in vitro techniques, as opposed to in vivo transfer to irradiated recipients, for the quantitation of helper cell activity we avoided the contribution of relatively radioresistant host cells (16) in the response. The use of DRC as a control for selective enrichment seemingly excludes the possibility of binding of CGG by the Fc receptors on helper cells (17).

The finding that a higher number of cells from the helper RFC-depleted population displayed some helping activity correlates to the in vivo findings. Radioiodinated antigen suicide abolished helper activity but the effect was overcome by higher doses of "suicidiced" helper cells (3). Both of these findings may be related to a low antigen-binding affinity by T helper cells.

Summary

The spleen T cells from mice immunized 6 days earlier with either chicken gamma globulin (CGG) or with donkey erythrocytes (DRC) were rosetted with CGG-coated sheep erythrocytes or with DRC. The immune rosettes (RFC) (antigen-binding cells) were separated from the bulk of nonrosette-forming cells (non-RFC) by 1-*g* velocity sedimentation and the RFC and non-RFC tested for helper activity in cooperative antihapten responses in vitro. RFC or non-RFC were mixed with normal or hapten-primed spleen cells, challenged with the appropriate hapten-carrier conjugate and cultured for 4 days in Marbrook tissue cultures. The helping activity was quantitated from the numbers of antihapten antibody-producing cells generated per culture.

The results show that specific helper cell activity could be selectively recovered in the immune rosette-forming cell population whereas the non-RFC population was depleted of help. These findings indicate that the helper T cells express specific antigen binding receptors.

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