Autologous Rosette-forming T Cells as the Responding Cells in Human Autologous Mixed-Lymphocyte Reaction

RONALD PALACIOS, LUIS LLORENTE, DONATO ALARCÓN-SEGOVIA, ALEJANDRO RUÍZ-ARGUELLES, and EFRAÍN DÍAZ-JOUANEN, *Department of Immunology and Rheumatology, Instituto Nacional de la Nutrición, México 22, D. F., México*

ABSTRACT Autologous rosette-forming cells (T*ar* cells) have surface and functional characteristics of post-thymic precursors and among these characteristics there are some that have been identified in the responsive cell of the autologous mixed-lymphocyte reaction (AMLR). We therefore did AMLR with circulating mononuclear cells from normal subjects using as responding cells either total T cells, T cells depleted of T*ar* cells, or purified T*ar* cells.

The response of T*ar* cells in AMLR was significantly greater than that of total T cells and these responded significantly more than T*ar*-depleted T cells. Conversely, T*ar* cells responded less than total T cells or T cells depleted of T*ar* cells in allogeneic mixed-lymphocyte reactions.

Increasing numbers of T*ar* cells gave significantly greater AMLR responses both alone and when added to diminishing proportions of T*ar*-depleted T cells to keep the number of T cells constant in the system.

T*ar* cells are the responding cells in AMLR but not in allogeneic mixed-lymphocyte reactions.

INTRODUCTION

Normally there is autoreactivity between subpopulations of lymphocytes. T lymphocytes respond to autologous non-T lymphocytes in coculture by proliferating (1). This phenomenon has been termed autologous mixed-lymphocyte reaction (AMLR)[1] and is impaired in the circulating mononuclear cells (MNC) from patients with systemic lupus erythematosus (2, 3).

We have recently determined that autologous rosette-forming T cells (T*ar* cells) have surface and functional characteristics of post-thymic precursors.[2] Among these characteristics there are several that have also been found to be those of the responding cells in AMLR (4–11). In this study we tested the role of T*ar* cells in AMLR and allogeneic mixed-lymphocyte reaction using MNC from young healthy adults and found them to be essential for the blastogenic response in AMLR but not in allogeneic mixed-lymphocyte response.

METHODS

Cells separation and identification. We obtained peripheral blood circulating MNC from healthy volunteers whose age ranged from 21 to 36 yr. We separated MNC in Ficoll-Hypaque (Pharmacia, Uppsala-Winthrop de México, Mexico City) gradients as described (12). From them we separated T cells and non-T cells also as described (12). Briefly, we incubated MNC with sheep erythrocytes (SRBC) at a ratio of 1:70, at 37°C for 15 min, spun them at 1,200 rpm for 10 min, and incubated them at 4°C for 18 h. We then relayered the cells' mixture on Ficoll-Hypaque, centrifuged at 1,400 rpm at 4°C for 30 min, and separately collected the rosetted cells in the pellet (total T cells) and the unrosetted cells at the interface (non-T cells). We lysed the SRBC-rosetting with total T cells by hypotonic shock. Total T cells included ≥94% SRBC-rosetting cells, <2% immunoglobin-bearing cells identified with fluorescein-labeled $F(ab')_2$ fragments of goat anti-human immunoglobulins serum (N. L. Cappel Laboratories Inc., Cochranville, Pa.), and <2% nonspecific esterase-positive monocytes (13). Non-T cells included ≥76% immunoglobin-bearing cells, ≤12% monocytes, and <1% T cells. We identified T*ar* cells as described.[2] Briefly, we placed 2×10^6 MNC in 0.2 ml of minimum essential medium (MEM) (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) and 0.05 ml of autologous serum diluted 1:5 in MEM and incubated them, 30 min at 4°C. We then added 16×10^6 autologous erythrocytes obtained from the same heparinized blood sample and washed thrice with phosphate-

Address reprint requests to Dr. Donato Alarcón-Segovia.
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[1] *Abbreviations used in this paper*: AMLR, autologous mixed-lymphocyte reaction; MEM, minimum essential medium; MNC, peripheral blood mononuclear cells; T*ar*, autologous rosette-forming T cells; T-T*ar*, T cells depleted of T*ar* cells; SI, stimulation index.

[2] Palacios, R., D. Alarcón-Segovia, L. Llorente, A. Ruíz-Arguelles, and E. Díaz-Jouanen. Human postthymic precursor cells in health and disease. I. Characterization of the autologous rosette-forming T cells as postthymic precursors. Submitted for publication.

buffered saline and resuspended in 0.8 ml of MEM. We centrifuged the cells' mixture 5 min at 1,000 rpm and incubated it overnight at 4°C. We resuspended the cells gently with Pasteur pipettes for either counting the T*ar* cells or for their separation on Ficoll-Hypaque gradients. For counting we placed the cells mixture on a hemocytometer Neubauer chamber and counted 300 lymphocytes. We counted as T*ar* cells those lymphocytes forming rosettes with three or more autologous erythrocytes. For separation we centrifuged the cells resuspended in cold MEM on Ficoll-Hypaque cushions at 4°C, 1,400 rpm for 30 min. We collected the rosetted cells in the pellet, resuspended them again in cold MEM, and repeated the Ficoll-Hypaque gradient separation at 4°C, 1,400 rpm for 30 min. We then resuspended the rosetted cells in the bottom in warm (37°C) MEM, incubated them 20 min at 37°C, and placed them on warm Ficoll-Hypaque and centrifuged them at 1,200 rpm for 20 min to rid them of the autologous erythrocytes. The T*ar* cells obtained had ≥94% autologous rosette-forming cells, <4% Tμ cells, identified by their rosetting with immunoglobin (Ig)M coated ox erythrocytes,[2] <1% Tγ cells, identified by rosetting with IgG coated ox erythrocytes (14), and <1% monocytes as identified morphologically and by nonspecific esterase staining.

We depleted total T cells from T*ar* cells by rosetting them with autologous erythrocytes and repeating the above procedure of gradient separation but collecting the non-rosetting T cells at the interphase (T-T*ar* cells). Total T cells included 30.8±3.5% T*ar* cells, whereas T-T*ar* cells had <2% T*ar* cells.

Autologous and allogeneic mixed-lymphocyte cultures. We incubated the separated MNC subpopulations overnight in MEM with 20% pooled normal human serum at 37°C in a 5% CO_2, 100% humidity atmosphere. We established mixed-lymphocyte cultures in triplicate, placing 1×10^5 of the separated T cell subpopulations as responding cells in 0.1 ml of MEM enriched with 20% pooled, heat-inactivated normal human serum, 0.8 mg L-glutamine (Sigma Chemical Co., St. Louis, Mo.), and 10 μg/ml of gentamicin (Schering Corp., Kenilworth, N. J.) and 1×10^5 of autologous or allogeneic stimulator non-T cells pretreated with 3 μg of mitomycin C (Sigma Chemical Co.) per 1×10^6 cells during 30 min at 37°C in a 5% CO_2, 100% humidity chamber. After this, we washed them thrice with MEM and resuspended in enriched MEM at a concentration of 1×10^6/ml. All cultures were done in culture plates with flat-bottom wells (Costar, Data Packaging, Cambridge, Mass.).

Isolated T cell subpopulation used as responding cells were placed in the same numbers and conditions unless otherwise indicated. We incubated all cultures 144 h at 37°C in a 5% CO_2, 100% humidity environment. 20 h before the termination of the incubation period we added 0.5 μCi of [*methyl*-^{3}H]thymidine (New England Nuclear, Boston, Mass.). At the end of the incubation period we harvested the cells in a multiple sample harvester, placed them in a standard scintillation mixture (New England Nuclear) and counted their thymidine incorporation in a liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

Results are reported as Δ counts per minute. (The difference of the counts per minute from cultures containing both responding and stimulator cells minus the counts per minute from cultures containing responding cells only). We also present them as stimulation indices (SI), which represent the ratio of the mean counts per minute in stimulated cultures over the mean counts per minute in unstimulated cultures.

Statistical analysis. Significance of our results was determined by paired Student's *t* test (two tailed) and by F variance test.

RESULTS

When we placed T-T*ar* cells as responding cells in the AMLR the response was significantly lower than that obtained when we placed total T cells. Conversely, when we placed isolated T*ar* cells, the response was significantly higher than that obtained with either total T cells or T-T*ar* cells.

In Table I we present the results of individual experiments and of their means, expressed both as Δ counts per minute as well as by their SI.

Gradual increase in the number of T*ar* cells placed as responding cells in the AMLR resulted in increase of the response obtained in the AMLR. These results were highly significant statistically with a 99% level of confidence in a F variance test (Fig. 1).

When we changed the proportion of T*ar* cells and

TABLE I

Tar Cells as Responding Cells in AMLR. Results of Individual Experiments and Their Means.

Experiment No.	Stimulator cells	Responding cells: Total T Δcpm	Total T SI*	T-Tar Δcpm	T-Tar SI	Tar Δcpm	Tar SI
1	non-T cells	3,229	7.68	860	1.5	7,640	18.1
2	non-T cells	2,892	8.35	678	1.4	4,654	13.4
3	non-T cells	2,710	7.97	936	1.9	4,503	13.2
4	non-T cells	2,525	6.97	741	1.6	5,141	14.2
5	non-T cells	3,548	10.60	987	2.1	5,372	16.0
6	non-T cells	3,140	7.65	796	1.8	4,962	12.3
mean±SEM*		3,067±157	8.2±0.5	833±48	1.7±0.1	5,379±472	14.5±0.8

* All differences significant at the $P < 0.0005$ level, paired Student's *t* test.

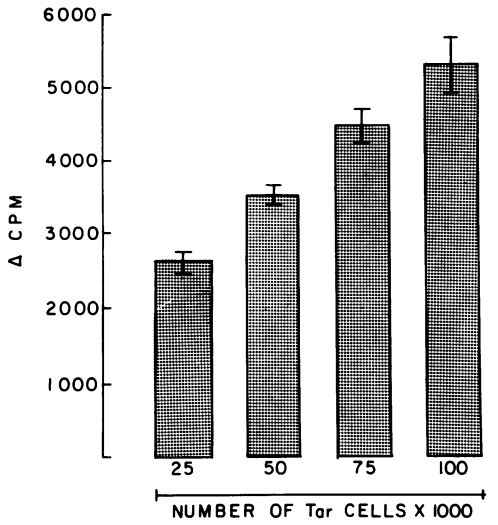

FIGURE 1 Blastogenic response in AMLR of increasing numbers of isolated T*ar* cells (mean±SEM of six experiments).

T-T*ar* cells in the AMLR we found increased responses as we increased the proportion of T*ar* cells despite both the simultaneous decrease of T-T*ar* cells and the keeping of a constant number of "responding" cells (Fig. 2). The results were also highly significant statistically at a 99% confidence level in a F variance test.

We found that T-T*ar* cells gave significantly higher responses in allogeneic mixed lymphocyte reaction than did total T cells or T*ar* cells (SI, mean of six experiments±SEM, T-T*ar* cells: 12.5±2.5, total T cells: 9.5±1.5, T*ar* cells: 3.7±1.0; all differences $P < 0.005$).

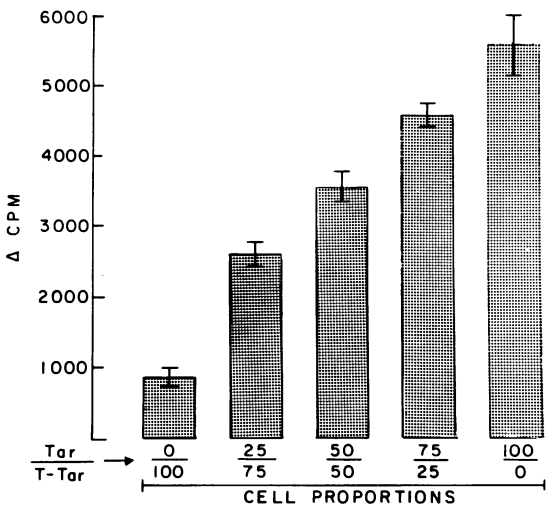

FIGURE 2 Effect of variations in the proportion of T*ar*/T-T*ar* responding cells in the blastogenic response in AMLR. The number of responding and stimulator (non-T) cells remained constant (1×10^5) (mean±SEM of six experiments).

Thus the responding cells in AMLR seem to be different from those responding in allogeneic mixed-lymphocyte reaction.

DISCUSSION

We have previously characterized T*ar* cells as postthymic precursors.[2] These medium-sized lymphocytes adhere to nylon wool, are theophylline resistant, have high affinity for SRBC, are nonspecific esterase-positive, are highly sensitive to hydrocortisone both in vitro and in vivo, and are devoid of receptors for the Fc portion of IgG or IgM. T*ar* cells respond to suboptimal doses of concanavalin A as well as to serum thymic factor but are unresponsive to phytohemagglutinin P or to pokeweed mitogen. Among them there is a subpopulation that binds peanut agglutinin (26.9±2.0%) indicating their immaturity. These characteristics permitted us to differentiate T*ar* cells from Tγ or Tμ cells. Functionally T*ar* cells are also peculiar because they participate in feedback inhibition and in the generation of suppression[2] and are precursors of natural killer cells.[3]

In this paper we show that T*ar* cells are the responding cells in AMLR, in that their removal from total T cells renders these irresponsive in AMLR. Also, purified T*ar* cells give significantly higher responses than do total T cells and their response increases linearly with increasing proportions of T*ar* cells. Conversely, T*ar* cells were found to have little or no role in the response in allogeneic mixed-lymphocyte reactions.

Our findings are in agreement with previous observations on the responding cells in AMLR. Thus, they have been determined to lack receptors for the Fc portion of IgG (4, 5), to be sensitive to hydrocortisone and responsive to concanavalin A (5–8), to be capable of generating suppression under concanavalin A stimulation (7, 8), and of generating natural cytotoxicity (9–11).

Peripheral blood MNC from systemic lupus erythematosus patients give low responses in AMLR (2, 3, 13). Accordingly, we have found quantitative and qualitative defects of circulating T*ar* cells in this disease.[4]

The AMLR is an interesting phenomenon that has both memory and specificity (15) and may reflect in vitro the operation of the immunoregulatory network. The finding of the responding cell in this system

[3] Palacios, R., D. Alarcón-Segovia, L. Llorente, A. Ruíz-Arguelles, and E. Díaz-Jouanen. Human postthymic precursor cells in health and disease. III. Autologous rosette-forming T cells are precursors of natural killer cells. Submitted for publication.

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and the feasibility of its isolation may help further understanding of immunoregulation and of the diseases of man where aberrations of its network occur (16).

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