

## **Autologous rosette-forming T cells regulate responses of T cells. Phenotypic and functional analysis of suppressor cells generated from autologous rosette-forming T cells after autologous mixed lymphocyte reactions.**

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**Research Article**

An average of 5--9% of human peripheral blood of T lymphocytes from rosettes with autologous erythrocytes (ARFT). This population responded only slightly against autologous and allogeneic non-T cells. In contrast, T cells that did not form rosettes with autologous erythrocytes (NRFT) proliferated to a greater degree in auto- and allogeneic mixed lymphocyte reactions (MLR) and also in reactions to trinitrophenyl (TNP) modified autologous non-T cells (TNP-auto-MLR) as compared with ARFT or unfractionated T cells. The ARFT populations could suppress the increased allogeneic (allo)MLR and TNP-auto-MLR of NRFT when the ARFT were added to the NRFT at the beginning of the cultures. Fluorescence-activated cell-sorter (FACS) analysis of these freshly obtained T cell fractions using monoclonal antibodies to subpopulations of T cells did not demonstrate any selective gain or loss of T cell subsets in the ARFT and NRFT as compared with unfractionated T cells. But when each T cell fraction was cultured separately for a week in the presence of autologous non-T cells (auto-MLR) and the cells were again analyzed by fluorescence-activated cell sorter, there was an increase in OKT8-positive cells (suppressor/cytotoxic subset) only in the ARFT fraction. The above findings strongly suggest that suppressor T cells are generated from the ARFT fraction during an auto-MLR, these may then regulate the responses on NRFT.

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# Autologous Rosette-forming T Cells Regulate Responses of T Cells

## PHENOTYPIC AND FUNCTIONAL ANALYSIS OF SUPPRESSOR CELLS GENERATED FROM AUTOLOGOUS ROSETTE-FORMING T CELLS AFTER AUTOLOGOUS MIXED LYMPHOCYTE REACTIONS

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**ABSTRACT** An average of 5–9% of human peripheral blood of T lymphocytes form rosettes with autologous erythrocytes (ARFT). This population responded only slightly against autologous and allogeneic non-T cells. In contrast, T cells that did not form rosettes with autologous erythrocytes (NRFT) proliferated to a greater degree in auto- and allogeneic mixed lymphocyte reactions (MLR) and also in reactions to trinitrophenyl (TNP) modified autologous non-T cells (TNP-auto-MLR) as compared with ARFT or unfractionated T cells. The ARFT populations could suppress the increased allogeneic (allo)MLR and TNP-auto-MLR of NRFT when the ARFT were added to the NRFT at the beginning of the cultures.

Fluorescence-activated cell-sorter (FACS) analysis of these freshly obtained T cell fractions using monoclonal antibodies to subpopulations of T cells did not demonstrate any selective gain or loss of T cell subsets in the ARFT and NRFT as compared with unfractionated T cells. But when each T cell fraction was cultured separately for a week in the presence of autologous non-T cells (auto-MLR) and the cells were again analyzed by fluorescence-activated cell sorter, there was an increase in OKT8-positive cells (suppressor/cytotoxic subset) only in the ARFT fraction. The above findings strongly suggest that suppressor T cells are generated from the ARFT fraction during an auto-MLR, these may then regulate the responses of NRFT.

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## INTRODUCTION

The discovery in the early 1970's that a very high proportion of human thymus-derived lymphocyte population formed spontaneous rosettes with sheep erythrocytes (SRBC)<sup>1</sup> became one of the important early bases for the identification and isolation of human T cells (1). In the course of studies of the binding properties of erythrocytes of other species to human lymphocyte populations, it was noted that a small proportion of human lymphocytes also formed rosettes with human erythrocytes (2–5). These lymphocytes were also identified as T cells. The next logical step was to determine whether this small fraction of T cells that formed rosettes with human erythrocytes constituted a subset of T cells with special properties and functions. Several recent studies have reported that these cells are less mature and have specialized functions (6–8).

Another interesting immunological phenomenon is the autologous mixed lymphocyte reaction (auto-MLR) and its role in the regulation of the immune response. The auto-MLR is an immunological reaction in which T

<sup>1</sup> *Abbreviations used in this paper:* Allo-MLR, allogeneic mixed lymphocyte reaction; ARFT, T cells forming rosettes with autologous human erythrocytes; auto-MLR, autologous mixed lymphocyte reaction; Con A, concanavalin A; HBSS, Hanks' balanced salt solution; NRFT, T cells not forming rosettes with autologous human erythrocytes; RBC, erythrocytes SRBC, sheep erythrocytes; T cells, thymus-derived lymphocytes; TNP, trinitrophenyl; TNP-auto-MLR, T cell response against TNP-modified autologous non-T cells; UT, unfractionated T cells.

cells proliferate in response to autologous non-T cells (9, 10). This reaction has been shown to have both immunological specificity and memory (11). Previous studies have suggested that T cells that proliferate in the auto-MLR may be preferentially able to develop suppressor or regulatory activities (12–15).

To explore the relationship between these two phenomena we performed the following series of experiments. First, we have examined various populations of T cells, unfractionated T cell (UT), T cells which formed rosettes with autologous human erythrocytes (ARFT), and T cells not forming autorosettes (NRFT) for their ability to react to auto-MLR and at the same time for their ability to respond to trinitrophenyl-modified autologous non-T cells (TNP-auto-MLR) and allogeneic non-T cells (allo-MLR). Populations of T cells depleted of ARFT (namely, the NRFT population) responded best to all of the above stimuli. Second, we have also examined the surface phenotypes of UT, ARFT, and NRFT using the OK series of monoclonal antibodies (16–19) before and after incubation with autologous non-T cells. Only the ARFT fraction developed an increase in percentage of cells with a suppressor phenotype after incubation in an auto-MLR. Finally, we have tested the capacity of the ARFT to act as suppressor cells; only the ARFT fraction developed suppressor activity against several responses of NRFT.

We thus demonstrate that although the ARFT proliferates poorly in the auto-MLR it is the only fraction that can develop both suppressor activity and the surface phenotype characteristic of suppressor cells.

## METHODS

**Separation of T cells, non-T cells, and monocytes.** Heparinized peripheral blood from healthy adult human donors was obtained from the Blood Bank Department (Clinical Center, National Institutes of Health, Bethesda, Md.). The blood was then diluted with an equal volume of Hanks' balanced salt solution (HBSS). 40 ml of the diluted blood was layered over 10 ml of Ficoll-Hypaque solution (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) and centrifuged for 30 min at 400 g. The mononuclear cells were then removed from the interface and washed three times in HBSS.

The T cells and non-T cells were then separated as previously described (20). In brief, T cells were recovered after double rosetting with neuraminidase-treated SRBC. This population consisted of >98% cells that rosette with SRBC and is called purified UT. The interface cells, obtained after the second cycle of the T cell purification procedure, were incubated overnight in petri dishes at 37°C. After this procedure, the nonadherent cells collected are designated as non-T cells. T cell contamination in the non-T cell preparations was <1%, as determined by rosetting with SRBC, and 10–20% of this population was identified as monocytes after Giemsa staining. This non-T cell population was used as the stimulator cells for auto- and allo-MLR. The adherent non-T cells were removed by a rubber police-

man, and >90% of these cells were identified as monocytes after Giemsa staining. These monocytes were added to the cultures in which the response of highly purified T cells to mitogens was being tested as described below.

**Fractionation of T cells into ARFT and NRFT.** Purified UT were rosetted with autologous human erythrocytes (RBC) with a modification of the technique of Kaplan (4). RBC were obtained from the same heparinized blood sample from which the mononuclear cells were obtained, washed four times with HBSS and resuspended in HBSS at a concentration of  $2 \times 10^8$ /ml.  $10^6$  purified UT were suspended in 1 ml of HBSS, and 2 ml of the above RBC suspension was mixed in a plastic tube (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif., cat. no. 2057) with 1 ml of heat-inactivated fetal calf serum, and incubated at 37°C for 10 min. The mixture was then centrifuged at 150 g for 5 min, and the tube was incubated at 4°C for 90 min.

The supernate was gently removed and the pellet in the tubes was mixed using a rotary shaker at 180 cycles/min for 3 min in a cold room at 4°C. Aliquots of this pellet were transferred to a chilled hemocytometer and the percentage of ARFT was determined; a lymphocyte with three or more attached RBC was considered a rosette. This cell suspension was then layered on cooled Ficoll-Hypaque and centrifuged for 15 min at 400 g. The nonrosetted interface cells (NRFT) and the rosetted cells at the bottom (ARFT) were collected separately and washed once with warm HBSS. These two populations were then suspended in 0.83% ammonium chloride-0.17 M Tris buffer (pH 7.2) to lyse RBC, centrifuged, washed three times in HBSS, and then resuspended in RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) supplemented with 100 U penicillin/ml, 100 µg streptomycin/ml, 2 mM L-glutamine, 25 mM HEPES buffer, and 5% heat-inactivated autologous plasma (the complete medium). UT, ARFT, and NRFT were cultured overnight at 37°C in complete medium in plastic petri dishes. The nonadherent cells were collected on the following day. Since the ARFT were at times contaminated by some remaining autologous RBC, the ARFT were layered again on Ficoll-Hypaque and spun for 15 min at room temperature to remove any nonlysed RBC.

**Auto- and allo-MLR.** UT, ARFT, and NRFT were used as the responder cells. The stimulator cells were non-T cells that had been obtained either from the same donor as the responding T cells or from an unrelated donor and incubated with 40 µg/ml of mitomycin C (Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37°C and washed three times in HBSS. Mixed lymphocyte cultures were established in triplicate using microtiter plates (Falcon Labware, cat. no. 3040) and consisted of 0.2 ml of the complete medium containing  $1 \times 10^6$  responding T cells and  $2 \times 10^4$  stimulating non-T cells. All cultures were incubated for 7 d at 37°C in a 5% CO<sub>2</sub> atmosphere. 20 h before harvesting, 1 µCi of [<sup>3</sup>H]methylthymidine ([<sup>3</sup>H]thymidine) (5 Ci/mol; Amersham Corp., Arlington Heights, Ill.) was added to each culture well. At the end of the incubation period the cells were processed on a microharvester, and incorporation of [<sup>3</sup>H]thymidine was measured by liquid scintillation counting. All data with regard to proliferative responses are expressed as the difference ( $\Delta$ cpm) between the counts per minute from cultures containing stimulating cells and the counts per minutes from cultures containing responding T cells alone. In some cases ARFT cells were added to the auto-MLR, allo-MLR, and TNP-auto-MLR (see below) of NRFT to determine whether such added ARFT could act as suppressor cells. The details of such experiments are presented in Results.

**Responses against TNP-modified autologous non-T cells.** Modification of stimulator non-T cells with trinitrobenzene

sulfonic acid was accomplished by the method used by Shearer et al. (21) for murine lymphocytes. Briefly,  $5-10 \times 10^6$  non-T cells/ml, after treatment with  $40 \mu\text{g/ml}$  of mitomycin C for 20 min, were incubated without washing for 10 min at  $37^\circ\text{C}$  with  $3.3 \text{ mM}$  trinitrobenzene sulfonic acid (Pierce Chemical Co., Rockford, Ill.) in HBSS titrated to pH 7.8 with  $1 \text{ N}$  NaOH. The modified cells were washed three times with HBSS to remove all unreacted trinitrobenzene sulfonic acid. Cultured conditions were exactly the same as those used for allo-MLR or auto-MLR. 20 thousand TNP-modified autologous non-T cells were added to  $1 \times 10^5$  UT, ARFT, or NRFT and cultured for 7 d. Net proliferative responses against TNP-modified cells (TNP-specific response) were calculated as follows: TNP-specific response =  $\Delta\text{cpm}$  of response against TNP-modified cells -  $\Delta\text{cpm}$  of response against nonmodified cells.

**Response of UT cells, ARFT, and NRFT to mitogens.**  $10^5$  of each type of responder T cell were mixed with optimal concentrations of phytohemagglutinin (Wellcome Foundation, Ltd., Beckenham, England) or concanavalin A (Con A; Pharmacia Fine Chemicals, Uppsala, Sweden) in 0.2 ml of RPMI-1640 supplemented with 20% fetal calf serum. In addition, since monocytes are necessary for the response of T cells to mitogens,  $1 \times 10^4$  mitomycin C ( $30 \mu\text{g/ml}$ )-treated monocytes were added to each well of the microtiter plate. In preliminary time-course studies, it was observed that UT, ARFT, and NRFT gave a maximal response to phytohemagglutinin and Con A in 72 h. Therefore, all mitogen stimulation experiments were harvested in 72 h, and incorporation of [ $^3\text{H}$ ]thymidine was measured as described above.

**Cytofluorographic analysis.** Cytofluorographic analysis of all cell populations was performed by indirect immunofluorescence. In brief,  $1-2 \times 10^6$  cells were treated with one of several monoclonal antibodies specific to different T cell subsets, termed OKT3, OKT4, and OKT8 (kindly provided by Dr. G. Goldstein, Ortho Pharmaceutical Corp., Raritan, N. J.), at a 1:200 final dilution, incubated at  $4^\circ\text{C}$  for 30 min, and washed three times. OKT3 identifies most T cells, OKT4 identifies helper/inducer T cells, and OKT8 identifies suppressor/cytotoxic T cells (16-19). The cells were then mixed with a 1:40 dilution of the developing antibody, a fluorescein-conjugated goat anti-mouse IgG (Meloy Laboratories Inc., Springfield, Va.). After washing, the fluorescence of the cells was measured using a fluorescent-activated cell sorter (FACS II, Becton, Dickinson & Co., East Rutherford, N. J.) and the intensity of fluorescence per cell was recorded on a pulse height analyzer. Indifferent monoclonal antibodies were used for background staining.

**Statistical analysis.** Student's *t* tests or paired sample *t* tests were used for analysis and the results are shown in the appropriate footnotes and figure legends.

## RESULTS

**Frequency and efficiency of recovery of ARFT.** In 14 individuals the average frequency of ARFT found in a population of twice SRBC-rosetted purified T cells was between 5 and 9% (determined by direct counting in a hemocytometer). In the ARFT fraction >90% of cells formed rosettes with autologous RBC; <2% of cells in the NRFT fraction formed rosettes with autologous RBC.

**Characteristics of ARFT and NRFT.** To determine whether any non-T cells were still present in the ARFT and in the NRFT fractions, in selected experi-

ments these populations were again rosetted with SRBC. More than 95% of each of these populations formed rosettes with SRBC.

We also wished to determine whether any of these T cell fractions were particularly enriched in T cells that have Fc receptors for IgG (Ty cells). Rosette formation with ox cells coated with IgG antibody were used to measure the percentage of Ty cells in the ARFT and NRFT fractions (22). In three studies, the average percentage of Ty cells in the ARFT was 7.8 and 9.7% in the NRFT fraction. These values do not differ significantly from the percentage of Ty cells in UT ( $9.6 \pm 2.2\%$ , mean  $\pm$  SE, six cases).

**Auto-MLR using ARFT and NRFT as responding cells.** Proliferative responses in the auto-MLR of UT, ARFT, and NRFT against autologous non-T cells were studied in 14 individuals. As shown in Fig. 1, auto-MLR of UT, NRFT, and ARFT were  $6,163 \pm 685$ ,  $10,496 \pm 1,180$ , and  $1,281 \pm 260$  (mean  $\pm$  SE, 14 cases), respectively.

It should be noted that, in all 14 experiments, the NRFT responded most vigorously, the ARFC least vigorously, and the unfractionated T cells gave an intermediate response. These differences among three groups were significant (*P* values are shown in Fig. 1).

**Allo-MLR using ARFT and NRFT as responding cells.** Allo-MLR were also compared using UT, NRFT, and ARFT as responding cells obtained from seven normal subjects. The data shown in Fig. 2 in-

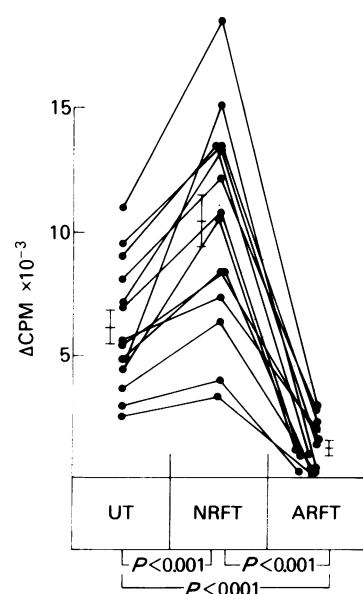


FIGURE 1 Proliferative response in auto-MLR of the UT, NRFT, and ARFT fractions in 14 individuals. The vertical bars show the mean  $\pm$  SE. *P* values of the differences among the groups are shown at the bottom of the figure. Calculated by paired sample *t* test.

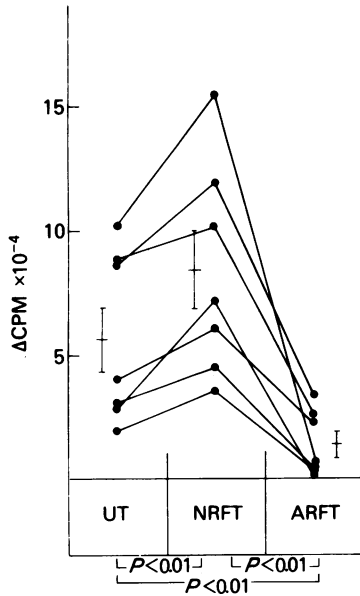


FIGURE 2 Proliferative response in allo-MLR of seven individuals. The vertical bars show the mean  $\pm$  SE. *P* values are shown at the bottom.

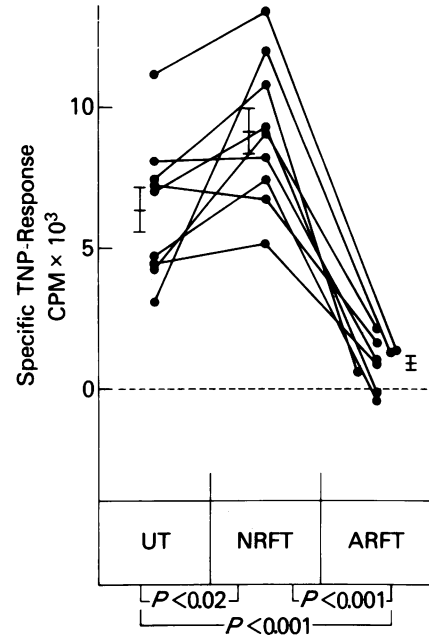


FIGURE 3 Proliferative response in TNP-auto-MLR of nine individuals. The vertical bars show the mean  $\pm$  SE. *P* values are shown at the bottom.

indicate that once again the NRFT gave the best response, the ARFT gave the least response, and the UT gave an intermediate response.

**Specific response to TNP-auto-MLR of UT, ARFT, and NRFT.** As shown in Fig. 3, T cells from nine individuals were studied. The TNP-specific response (Methods) is shown. Again, the NRFT fraction gave the greatest response, the ARFT the least response, and the UT gave an intermediate response. As evidence that these responses were TNP specific, aliquots of cells from the primary culture were tested for their secondary proliferative and cytotoxic response. A significant response was observed only against TNP-modified stimulator cells (data not shown).

**Mitogen responses of UT, ARFT, and non-ARFT.** To show that the ARFT fraction, which did not respond in auto-MLR, allo-MLR, and TNP-auto-MLR, could respond to some stimuli, we tested the proliferative responses of these T cell fractions to mitogens. Table

I shows the responses of UT, ARFT, and NRFT to an optimal concentration of phytohemagglutinin and Con A. No differences were observed in the responses among these three fractions.

**Cytofluorographic analysis.** UT, ARFT, and NRFT were studied for their surface antigens with cytofluorographic analysis with OKT3-, OKT4- and OKT8-monoclonal antibodies (16–19). As shown in Table II, freshly obtained UT, ARFT, and NRFT all showed equivalent percentages of OKT3-, OKT4-, and OKT8-positive cells. An aliquot of these cells were then placed in an auto-MLR; after culture for 7 d, the cells were harvested and again examined cytofluorographically. As shown in Table III, the percentage of OKT8-positive cells in these experiments increased only in the ARFT population. Fig. 4 shows a representative fluorographic plot of the clear increase in the percentage of OKT8-positive cells observed in the

TABLE I  
Mitogen Responses of UT, ARFT, and NRFT

Mitogen	Concentration $\mu\text{g/ml}$	Cells		
		UT	ARFT	NRFT
Phytohemagglutinin	1.0	71,478 $\pm$ 15,345*	51,139 $\pm$ 14,572	87,396 $\pm$ 13,373
Con A	25	68,047 $\pm$ 12,597	54,118 $\pm$ 11,419	85,755 $\pm$ 11,070

\* Values are the mean  $\pm$  SE of  $\Delta\text{cpm}$  of studies performed on lymphocytes from six individuals.

**TABLE II**  
Percentage of OKT3-, OKT4-, and OKT8-positive Cells in Freshly Obtained UT, ARFT, and NRFT Fractions

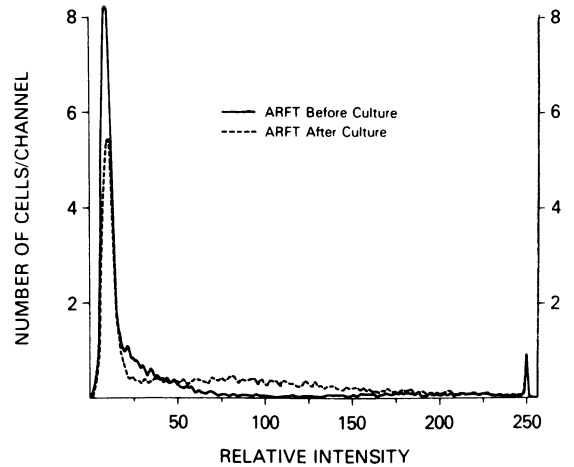
Cells	Antibody	Experiment				Mean±SE
		1*	2	3	4	
UT	OKT3	94.6†	90.0	94.4	ND‡	93.3±1.2
ARFT	OKT3	93.8	84.4	93.7	ND	90.6±3.1
NRFT	OKT3	94.0	90.9	94.4	ND	93.1±1.1
UT	OKT4	53.9	70.9	56.1	57.4	59.6±3.8
ARFT	OKT4	58.2	70.6	46.5	43.5	54.7±6.2
NRFT	OKT4	53.0	71.9	52.3	52.2	57.4±4.9
UT	OKT8	31.7	27.8	30.6	38.6	32.2±2.3
ARFT	OKT8	31.3	38.0	34.3	31.9	33.9±1.5
NRFT	OKT8	34.0	25.0	29.1	35.9	31.0±2.5

\* Each experiment represents studies on the lymphocytes of a different individual; they correspond with the lymphocytes used in the first four experiments shown in Table II.

† The number is the percentage of cells stained by the particular monoclonal antibody used with the FACS II.

‡ Not done.

ARFT fraction. In spite of this increase in OKT8-positive cells in the ARFT fraction, it should be noted that the percentage of OKT4-positive cells in this fraction did not decrease below what was observed in cells at the start of culture (Tables II and III).



**FIGURE 4** Fluorescent-activated-cell-sorter profile of ARFT stained by OKT8 before (—) and after (---) culture in auto-MLR.

To determine whether an auto-MLR was required for the change in the OKT8 phenotype observed, we cultured each cell fraction alone for 7 d and tested their phenotype; in three separate experiments no change in the proportion of cells staining with OKT3, OKT4, and OKT8 was noted.

*ARFT suppresses increased auto-MLR, allo-MLR, and TNP/auto-MLR of NRFT.* As previously shown in Figs. 1, 2, and 3, the response of NRFT was greater

**TABLE III**  
Percentage of OKT3-, OKT4-, and OKT8-positive Cells in UT, ARFT, and NRFT Fractions after 7 d of Culture in Auto-MLR

Type of responder T cells	Antibody	Experiment					Mean±SE
		1	2*	3	4*	5*	
UT	OKT3	ND†	ND	87.1	88.8	93.3	89.7±1.8
ARFT	OKT3	ND	ND	87.0	92.6	86.1	88.6±2.0
NRFT	OKT3	ND	ND	85.5	89.0	93.2	89.2±2.2
UT	OKT4	66.3‡	64.9	68.8	55.5	62.8	63.6±2.3
ARFT	OKT4	76.3	76.6	51.5	38.0	42.0	56.9±8.3
NRFT	OKT4	64.6	69.1	58.1	52.5	56.4	60.1±3.0
UT	OKT8	20.0	15.8	39.1	39.5	38.0	30.5±5.2
ARFT	OKT8	51.5	52.5	48.8	57.5	47.0	51.5 <sup>§</sup> ±1.8
NRFT	OKT8	17.0	18.2	33.1	39.8	41.3	29.9±5.2

\* In these experiments the cells obtained after 7 d were separated by rosetting with SRBC (exactly as in the method used to originally obtain T cells) before their analysis by FACS.

† Not done.

‡ The number is the percentage of cells stained by the particular monoclonal antibody used with the FACS II.

<sup>§</sup> This percentage is significantly different by Student's *t* test from the same fraction examined in Table II. It is also significantly different from the percentage of the OKT8 stained UT NRFT fractions.

against TNP-modified or unmodified autologous and allogeneic non-T cells than the response of unseparated T cells. To determine whether these increased responses of NRFT were the result of an absence of ARFT, ARFT were deliberately added to the auto-MLR, allo-MLR, and TNP-auto-MLR of NRFT at the beginning of the culture. The percentage of suppression at the point in the curves (Figs. 5-7) where suppression was maximal was calculated for each experiment as follows:

Percent suppression

$$= \left( 1 - \frac{(\Delta\text{cpm}) \text{ addition of ARFT}}{(\Delta\text{cpm}) \text{ no addition of ARFT}} \right) \times 100.$$

Fig. 5 indicates that addition of increasing numbers of ARFT to a constant number of NRFT suppressed the allo-MLR of NRFT in a linear fashion. The percentage of suppression ranged from 25 to 65% with a mean  $\pm$  SE of  $47.0 \pm 6.1\%$ .

As shown in Fig. 6, when ARFT were added to auto-MLR of NRFT, the increased auto-MLR of NRFT were suppressed almost linearly when  $0.63$ – $2.5 \times 10^4$  cells were added, but when  $5$ – $10 \times 10^4$  ARFT cells were added, no suppression was observed. At the point of maximal suppression ( $2.5 \times 10^4$  ARFT cells added) the suppression ranged from 31.2 to 78.6% with a mean  $\pm$  SE of  $51.3 \pm 5.2\%$ . As shown in Fig. 7, when increasing numbers of ARFT were added to the TNP-auto-MLR, a marked suppression of the response of NRFT was observed. The mean suppression observed was 95.5% when  $5 \times 10^4$  ARFT were added.

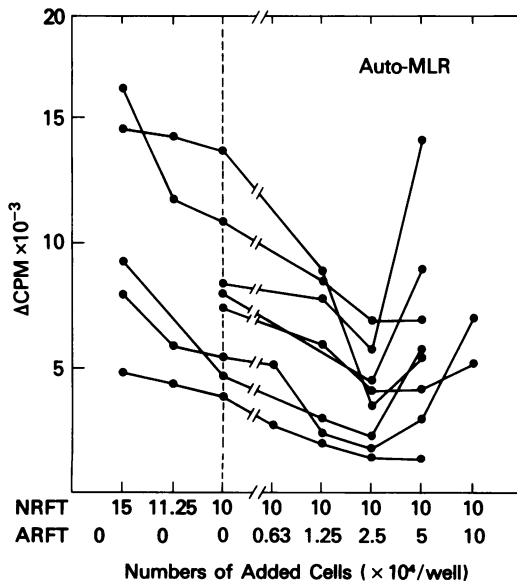


FIGURE 5 Effect of adding increasing numbers of ARFT to an allo-MLR of NRFT.

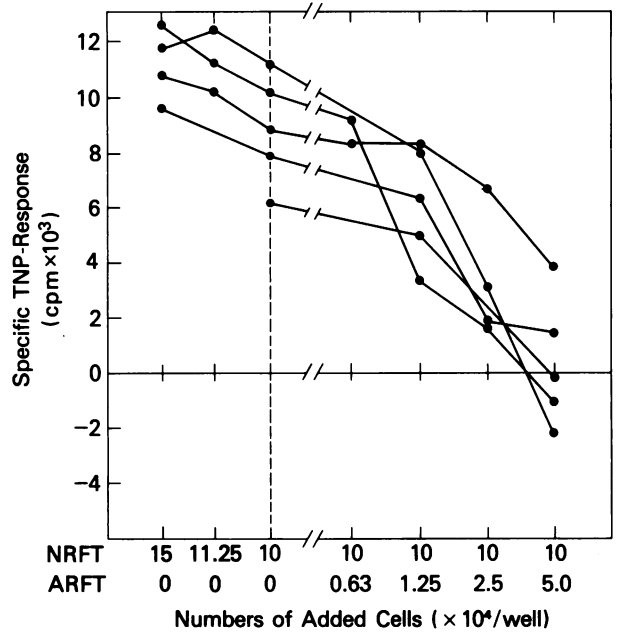


FIGURE 6 Effect of adding increasing numbers of ARFT to an auto-MLR of NRFT.

In these experiments the total number of cells in the cultures varied, and it is possible that the apparent inhibitor caused by the addition of ARFT might be due to crowding rather than a specific inhibition caused by the ARFT. In the left part of Figs. 5-7, we show the effect of having increasing numbers of NRFT in the cultures. Even with  $15 \times 10^4$  cells/culture no inhibition of proliferation was observed.

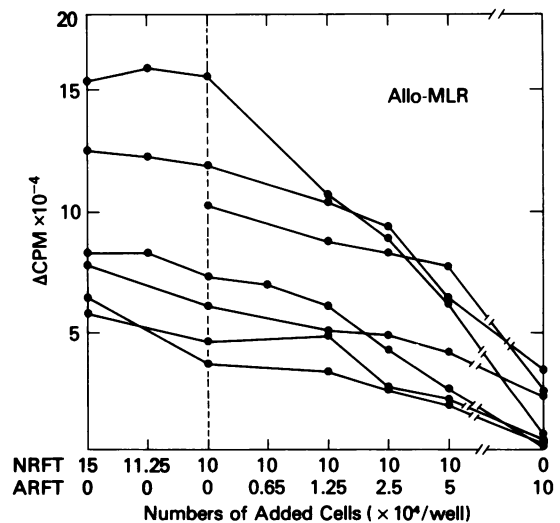


FIGURE 7 Effect of adding increasing numbers of ARFT to a TNP-auto-MLR of NRFT.

## DISCUSSION

In the present study, we demonstrate that an average of 5–9% of human T cells form rosettes with autologous erythrocytes (ARFT). The functional ability and surface phenotype of this subpopulation of T cells were compared with UT and T cells that did not form rosettes with autologous erythrocytes (NRFT). The ARFT population responded poorly in the auto- and allo-MLR as compared with the other populations of T cells. Moreover, this ARFT population could suppress the auto- and allo-MLR and TNP-auto-MLR of NRFT.

Studies of the surface phenotypes of freshly obtained UT, ARFT, and NRFT showed that >90% of the cells in each fraction stained with OKT3 indicating their T cell origin. Furthermore, there was no difference in the percentage of distribution of OKT3-, OKT4-, and OKT8-positive cells in any of these fractions of freshly obtained cells. After incubation in the auto-MLR, however, it was observed that the percentage of OKT8-positive cells increased only in the ARFT population.

It is unlikely that this increase in staining is due to Fc receptors on this subpopulation because the staining was performed at 4°C with reagents that were ultracentrifuged just before use. Furthermore, in several experiments these populations of T cells were also treated with OKM1; no staining was observed (data not shown).

Several other groups have described the properties of ARFT. The percentage of ARFT in human thymocytes is greater than in peripheral blood and exposure to thymic factor causes a decrease in ARFT (2–4, 23). It was also found recently by Palacios et al. (7) that ARFT bound peanut agglutinin and that ARFT were hydrocortisone sensitive. It has also been shown by Fournier and Charreire (23) that after exposure of T cells to Con A there is a dramatic increase in the number of ARFT. These various observations indicate that the ARFT form a less mature subpopulation of cells.

One of the problems in any discussion of ARFT is the fact that there is a considerable difference between the results of different authors in the percentage of these cells found in peripheral blood. In studies in which neuraminidase-treated RBC are used to form rosettes, 30–50% of T cells form ARFT (8); in other studies in which RBC are not neuraminidase treated, the percentage of ARFT in peripheral blood is <10% (2–5, 23). However, Palacios et al. (6, 7), using the latter technique, observed that 30% of T cells formed autorosettes.

Functional studies of these cells have been performed principally as to their role as responder cells in the auto- and allo-MLR. The auto-MLR occurs when T cells are exposed to autologous non-T cells (9, 10); this reaction has been shown to have both immunologi-

cal specificity and memory (11), and in several studies it has been reported that T cells stimulated in an auto-MLR develop Con A-induced or naturally occurring suppressor activity (12, 14, 15). Palacios et al. (6) reported that the ARFT were able to respond in the auto-MLR better than NRFT, but that in the allo-MLR the ARFT responded less well. Tomonari et al. (8) (using ARFT obtained with neuraminidase-treated RBC) observed that ARFT were the major subset that responded in the auto-MLR. Thus, with regard to the abilities of ARFT to respond in auto-MLR, these reports are at variance with our observations, as we observed that the NRFT population responded better than ARFT and UT in the auto-, allo-, and TNP-auto-MLR. The discrepancy between our results and those of Tomonari et al. (8) can be explained in part by differences in technique; their ARFT certainly include T cells not found in our ARFT population. The percentage of ARFT in peripheral blood obtained by Palacios et al. (6, 7) is also greater than what we observed in spite of rather similar technique; nevertheless, their ARFT fraction must also have contained cells not present in our fractions. Indeed, it should be noted that in our study (Fig. 6), when increasing numbers of ARFT were added to a constant number of NRFT and the ratio of ARFT increased above that present in normal peripheral blood, there was also an increase in the auto-MLR. This latter observation may be due to some type of T-T interaction.

Another function that we have studied is the ability of the ARFT to act as suppressor cells. Indeed, we observed that ARFT during an auto-MLR can suppress the auto-, allo-, and TNP-auto-MLR of responding NRFT. It was interesting that these functional studies were supported by the marked increase in percentage of OKT8-positive cells, a class of T cells previously shown to act as suppressor cells (17–19). If we calculate the absolute number of OKT8-positive cells in each fraction by multiplying the total number of recovered cells after auto-MLR (usually 70% of the starting number) by the percentage of OKT8-positive cells in each fraction, then it was observed that the absolute number of OKT8-positive cells increased slightly in ARFT fraction. There was, however, a decrease in the absolute number of OKT8-positive cells in NRFT or UT fraction. These findings are compatible with the fact that minimal proliferation was observed in the auto-MLR of ARFT. Nevertheless, only the ARFT fraction could act as a suppressor of the auto-MLR, TNP-auto-MLR, and allo-MLR, as demonstrated in our functional studies (Figs. 5–7). As evidence that the ARFT could acquire special characteristics despite their minimal proliferative response during a primary MLR is the fact that they can show a considerable proliferative response in a secondary auto-MLR.



Related to these above findings are a series of other observations. First, as mentioned previously, Con A exposure leads to an increase in the percentage of ARFT (23). Second, other data indicate that the T cells responding in the auto-MLR are especially able to develop Con A-induced suppressor activity (14, 15). Third, Palacios et al. (7) have also recently reported that ARFT could suppress pokeweed mitogen-induced immunoglobulin synthesis.<sup>2</sup> Thus, the ARFT appears to contain cells capable of mediating suppressor effects after activation either by an auto-MLR or by mitogens.

The ARFT population thus appears to be early in the series of T cell differentiation and contains both helper and suppressor cells, as defined by their reaction with OKT4- and OKT8-monoclonal antibodies (17–19). The ARFT population may be especially enriched with undifferentiated precursor cells that can give rise to a variety of functional T cells. Palacios et al. (7) have suggested that the ARFT might be the equivalent of an Ly123 and feedback suppressor cell (24, 25). Moreover, most human thymocytes have been shown by Reinherz et al. (17) to co-express T3, T4, T8, and other antigens. Although in the circulating blood OKT4 and OKT8 cells appear to be separate populations, it is possible that a small subpopulation of peripheral T cells carry both markers and such cells are concentrated in the ARFT (which are thought to be immature). These immature cells might have changed to OKT8-positive cells during an auto-MLR; proliferation may not be necessary for this process.

It is also of considerable interest that the phenomenon of autorosette-forming thymocytes can also be observed in the mouse (26, 27). In this species, however, the ability to form rosettes requires histocompatibility in the L region between the T lymphocyte and the mouse erythrocytes (28–30). No functional studies of the ARFT-forming T cell subset in the mouse have yet been reported.

There are suggestions that the frequency of ARFT in disease states may be altered. For example, Charreire and Bach (26, 27) reported that the frequency of ARFT was increased in New Zealand black, nude, and old mice. Sandilands et al. (3) reported an increase in ARFT in patients with melanoma, and Palacios et al. (6, 7) mentioned that the frequency of ARFT was decreased in patients with systemic lupus erythematosus (6, 7). We have recently observed that the anti-T cell antibodies found in patients with systemic lupus erythematosus are preferentially able to kill ARFT (data submitted for publication). In this

regard Huston et al. (31) have also observed that natural anti-T cell antibodies in New Zealand black mice preferentially killed immature thymocytes. Further studies of the frequency of ARFT in other disease states might be of considerable interest.

The phenomenon of ARFT formation may be a new way to isolate a subset of T cells with special properties; whether this phenomenon also occurs in vivo and has a function in vivo remains to be determined.

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