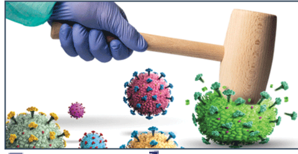
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Catherine Fournier; ... et. al

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ACTIVATION OF A HUMAN T CELL SUBPOPULATION BEARING RECEPTORS FOR AUTOLOGOUS ERYTHROCYTES BY CONCANAVALIN A

CATHERINE FOURNIER AND JEANNINE CHARREIRE

From INSERM U 25, Hôpital Necker, 161, rue de Sèvres, 75730 Paris, Cedex 15, France

Human lymphocytes from different lymphoid organs were examined for rosette formation with autologous erythrocytes. The autorosette-forming cells (A-RFC) were shown to belong to a T cell subset including less mature lymphocytes. When normal human peripheral blood lymphocytes were stimulated with low doses of the plant lectin concanavalin A (Con A), in the presence of autologous plasma, the A-RFC levels were strongly enhanced. This response gave rise to two peaks: the first one coincided with the peak of thymidine incorporation but the maximum increase occurred 5 or 6 days later when the proliferative response was impaired. Depletion of A-RFC before stimulation with Con A led to a clear-cut decrease in autorosette levels at both peaks of the response. It is concluded that Con A, generally used for polyclonal activation against heteroantigens, may also result, in terms of A-RFC marker, in expansion of an autoreactive T cell population.

In recent years, evidence has been accumulated that human lymphocytes are capable of binding autologous erythrocytes to form rosettes (1–5). We have previously shown that autorosette levels are higher in women than in men, and that they increase with age in both sexes. However, rosette levels never exceed 3% of peripheral blood lymphocytes (PBL).[1] Using E rosette depletion and mixed rosettes (with both autologous and sheep red blood cells), we have demonstrated the T cell origin of these autorosettes (6). The object of the present study is to investigate further the properties of the T cell subpopulation that forms autorosettes. The incidence of autologous rosette-forming cells (A-RFC) was studied among different lymphoid organs and their sensitivity to synthetic thymic extracts was assessed. Since the mitogen concanavalin A (Con A) has commonly been used to induce polyclonal activation against alloantigens (7–9), the same system was used to study lymphocytes that bind autologous erythrocytes.

MATERIALS AND METHODS

Reagents. Ficoll-Angiocontrix mixture for blood lymphocyte isolation was prepared with 24 volumes of 9% Ficoll (Pharmacia, Piscataway, N. J.) and 10 volumes of Angiocontrix (30% sodium iotalamate, Guerbet).

Thymic extracts. Purified synthetic thymic factor, which we term FTS (*facteur thymique sérique*), was prepared as previously described (10) and used at various concentrations ranging from 10^{-15} M to 10^{-9} M.

Cell isolation. Thymus cells were obtained from normal thymus tissue removed at the time of surgery to facilitate repair of congenital heart defects in children. Spleen and lymph nodes were surgical specimens from trauma victims. Tonsils were obtained from children undergoing elective tonsillectomy. All tissues were teased with forceps in Hanks' medium, filtered through a fine nylon mesh, and then washed twice in Hanks' medium.

Human blood from healthy donors was collected on heparin. It was then diluted 1/3 in Hanks' medium and layered on a Ficoll-Angiocontrix mixture. The tubes were centrifuged at 400 $\times$ G for 15 min. The cells at the interface were collected, pooled, and washed twice in Hanks' medium.

Autorosette test. 1.5×10^6 viable lymphocytes were mixed with various concentrations of autologous erythrocytes (obtained from blood and washed three times) in a total volume of 0.25 ml of Hanks' medium. The cell suspension was centrifuged for 5 min at 200 $\times$ G and incubated overnight at 4°C. The rosettes were then gently resuspended for 2 min on a rotating platform (10 rpm). Rosettes, defined as the binding of at least 3 red cells/lymphocyte, were read in a Malassez hemocytometer. Each determination was made in duplicate and after counting at least 3000 lymphocytes, the results were expressed as the number of A-RFC per thousand total lymphocytes. In the study of autorosette levels in different lymphoid organs, several autologous red blood cells to white cells (A-RFC:WC) ratios were used. In all other experiments the studies were performed on human PBL at optimal A-RFC:WC ratio (64:1).

E-rosette test. This test has previously been described (11). In brief, 15×10^6 fresh sheep erythrocytes were mixed with 5×10^5 mononucleated cells in Hanks' medium supplemented with 25% fetal calf serum previously absorbed with sheep and human erythrocytes. The cell mixtures were incubated for 30 min at 37°C, centrifuged for 5 min at 200 $\times$ G, and stored overnight at 4°C before being resuspended and read in a Malassez hemocytometer.

Surface immunoglobulin determination. Mononucleated cell suspensions were incubated at 4°C for 30 min with a fluorescence polyvalent anti-immunoglobulin serum (Behring, Marburg, Germany) and surface immunoglobulin (sIg)-bearing cells were scored.

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[1] Abbreviations used in this paper: A-RBC, autologous red blood cells; A-RFC, autologous rosette-forming cells; CFC, cluster-forming cells; FTS, *facteur thymique sérique*; PBL, peripheral blood lymphocytes; sIg, surface immunoglobulin; SLE, systemic lupus erythematosus; WC, white cells.

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Autorosette depletion. Autorosettes were set up in a RBC:WC ratio of 64:1 as described above, except that all volumes were multiplied by 2, and the incubation at 4°C did not exceed 2 hr. Aliquots of 8 ml of rosette suspensions were layered carefully on 4 ml of Ficoll-Hypaque mixture obtained by adding 10 volumes of 14% Ficoll to 24 volumes of 32.8% Hypaque (Nyegaard, Oslo). The tubes were centrifuged at room temperature for 15 min at 250 × G. The interface layers were collected, pooled, and washed twice in Hanks' medium.

Con A stimulation. Lymphocytes were suspended at a concentration of 10^6/ml in RPMI 1640 medium supplemented with 2 mM glutamin, 100 units/ml penicillin, 100 μg/ml streptomycin (Flow Laboratories, Rockville, Md.), and 20% inactivated autologous plasma. Unless specified, concanavalin A (Con A, Miles-Yeda, Israel) was added at a final concentration of 2 μg/ml of culture. Cells were distributed in volumes of 5 or 10 ml into plastic flasks (Falcon, Oxnard, Calif. No. 3013) and incubated for various periods at 37°C in a humidified atmosphere of 5% CO_2 in air. At the term of the culture, cells were washed twice in Hanks' medium and scored. Lymphocyte suspensions (containing 1.5×10^6 viable cells) were mixed with autologous erythrocytes (stored from day 0 at 4°C in Alsever solution) and autorosette formation was performed as described above.

Thymidine incorporation. Aliquots (0.2 ml) of cell suspension were pipetted from the flask at the end of the culture period and pulsed with 1 μCi of ^{3}H thymidine (C.E.A., Saclay, France) for 18 hr at 37°C in flat-bottomed microplates (Falcon No. 3014). Each determination was performed in triplicate. Cells were then harvested on an automated sample harvester and the amount of ^{3}H thymidine incorporation was evaluated by liquid scintillation counting. The results were expressed by the average values in counts per minute (cpm) of each sample.

RESULTS

Distribution of autorosettes in human lymphoid organs. In lymphoid organs, autorosette levels were assessed with various autologous red blood cells (A-RBC) to white cells (WC) ratios ranging from 4:1 to 128:1. Figure 1 shows the average values of 22 thymuses tested. The levels increased along with the A-RBC/WC ratios to reach 276.2 ± 38.2 A-RFC per thousand lymphocytes at the highest ratio. These levels were much greater compared to those obtained in other lymphoid organs. Indeed, less than 10 per thousand PBL were able to bind autologous erythrocytes whatever the ratio used. Slightly higher levels were obtained in the spleen, lymph node, and tonsils. These data confirm our previous reports (6) and suggest, in fact, that A-RFC represent a subset of less mature T lymphocytes.

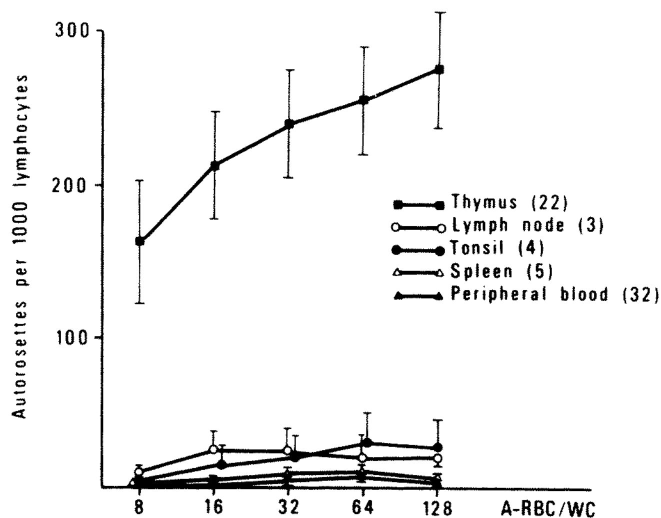

Figure 1. Autorosette levels in human lymphoid organs with different A-RBC:WC ratios. Mean ± S. E. The figures in parentheses indicate the number of lymphoid organs tested in each case.

Sensitivity of autorosettes to thymic extracts. In order to study further this last conclusion, the action of thymic extracts on autorosette formation in the thymus was assessed. In eight out of nine experiments, the *in vitro* incubation of thymocytes with synthetic FTS for 1 hr at 37°C led to a significant inhibition of 50 to 60% of autorosette levels for a concentration of 10^{-13} M (Table I). Similar results were obtained with PBL of aged subjects (since they have higher autorosette percentages than the younger adults).

Effect of Con A stimulation on autorosette formation. The action of Con A, a mitogen that induces the proliferation of both mature and less mature T cells (12–14), was studied on autorosette formation. For this purpose human PBL, suspended in appropriate medium supplemented with 20% inactivated autologous plasma, were stimulated by various Con A concentrations ranging from 0.5 μg/ml to 50 μg/ml (Table II). The number of autorosettes formed after a culture period of 4 days increased parallel to Con A concentrations. However, a final concentration of 2 μg/ml was shown to be optimal and reproducible since higher doses induced cell agglutination and therefore prevented satisfactory rosette evaluation.

In order to ascertain that the increase in A-RFC levels was true stimulation and not due to a nonspecific binding of the erythrocytes on Con A molecules at the cell surface, the effect of Con A was competitively blocked by addition of 0.05 M α-methyl-D-mannopyranoside 5 hr before the end of the culture (Table III). Under these conditions, the autorosette stimulation of human PBL remained unchanged in comparison to that obtained with lymphocytes with α-methyl-D-mannopyranoside. Moreover, when the same Con A concentration was added to unstimulated lymphocytes, before rosette formation, the autorosette levels were not modified.

TABLE I

Inhibition of thymus autorosettes after incubation in vitro for 1 hr at 37°C with synthetic FTS[a]

FTS Concentrations	A-RFC per Thousand Lymphocytes	
M		
0	295.3 ± 53.7	
10^{-15}	201.8 ± 56.8	$p < 0.05$
10^{-13}	141.2 ± 36.3	
10^{-11}	172.1 ± 39.1	
10^{-9}	169.5 ± 38.7	

[a] Mean ± S.E. of eight experiments (A-RBC:WC = 64:1).

TABLE II

Effect of Con A concentration on autorosette formation by human PBL after a 4-day culture[a]

Con A Concentrations	Autorosette Numbers (per Thousand Lymphocytes)
μg/ml	
0	3.5 ± 1.7
0.5	9.8 ± 3.8
2	36.1 ± 1.0
10	56.5 ± 16.5
50	55.4 ± 9.5

[a] Mean ± S.E. of seven experiments (A-RBC:WC = 64:1).

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Kinetic studies revealed that the autorosette stimulation was not immediately manifested but became marked on day 3 (Fig. 2). At that time, A-RFC percentages reached 10 to 15 times more than initial levels at zero time or control culture levels with unstimulated lymphocytes. A second response peak was observed from day 9 to day 13, with a rapid decline thereafter. It increased 35 to 40 times above background levels, although a weak augmentation of autorosettes was noted in unstimulated control cultures (Fig. 2).

Relationship between autorosette stimulation, proliferation response, and surface markers. The time course of mitogen-induced autorosettes was compared with the proliferation curve obtained in similar culture conditions (20% autologous plasma) with the same Con A concentration of 2 μg/ml, which is much lower than the usual mitogenic dose (50 μg/ml). Figure 3*B* shows the thymidine uptake produced in our culture conditions and the percentages of viable cells recovered related to the original cell numbers (*C*). The peak of these responses, reached on day 4 for DNA synthesis and on day 6 when cell recovery was measured, corresponds to the first autorosette stimulation (*A*). As shown in Figure 3*D*, at that time the percentage of dead cells present in the culture was about 10%. It began to increase gradually from day 7 and reached 30 to 40% when the second autorosettes peak occurred. In four experiments, it was verified that the majority of lymphocytes that bind autologous erythrocytes were not stained by trypan blue. The surface markers of Con A-stimulated cells were assessed by E rosette formation and determination of sIg-bearing cells to identify, respectively, T and B lymphocytes (Fig. 3*E*). No significant change in the proportion of T and B cells was noted during the stimulation although it declined slowly, simultaneously with the increase in the number of dead cells.

Effect of A-RFC depletion on Con A-stimulated autorosettes. Depletion experiments were monitored to investigate further whether Con A-stimulated autorosettes involved the cells forming spontaneous rosettes with autologous erythrocytes. Autorosette depletion was performed by centrifuging the lymphocyte suspension after autologous rosette formation on Ficoll-Hypaque gradient and collecting the cell ring at the interface. Control cell preparations, centrifuged in identical conditions but without previous rosette formation, did not show any significant change in autorosette number.

Initial and A-RFC-depleted preparations were cultured with 2 μg/ml Con A, and their ability to form autorosettes was examined on day 5 and on day 12, which represent the two main autorosette responses. Only experiments where the depletion index on day 0 was higher than 80 were considered (five

TABLE III

Con A-stimulated autorosettes in human PBL after competition with α-methyl-D-mannopyranoside (added 5 hr before the end of the culture)[a]

	Con A-Stimulated Autorosettes (per Thousand Lymphocytes)		
	Control cultures		Addition of 0.05 M α-methyl-D-mannopyranoside
Day 3	28.6 ± 12.2	N.S.[b]	36.4 ± 15.3
Day 6	24.6 ± 5.8	N.S.	16.4 ± 4.4
Day 9	49.7 ± 17.6	N.S.	33.5 ± 4.5

[a] Mean ± S.E. of seven experiments (Students *t*-test) (A-RBC: WC = 64:1).

[b] N.S., not significant.

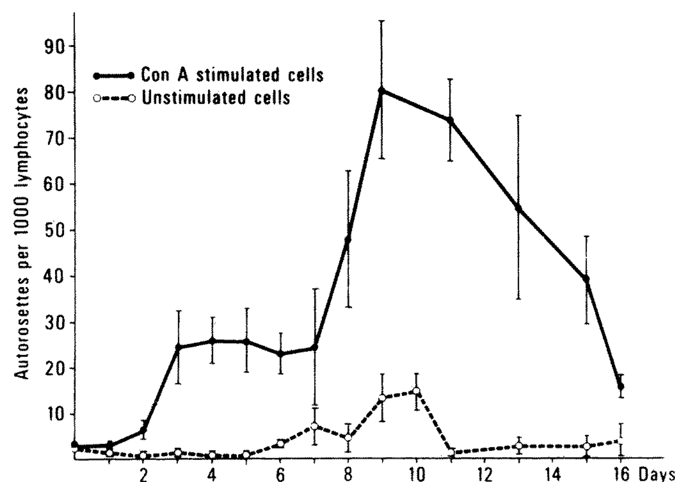

Figure 2. Kinetics of autorosette formation in human PBL after stimulation with 2 μg/ml Con A (●——●) or without Con A (○-----○). Mean ± S. E. of 10 experiments.

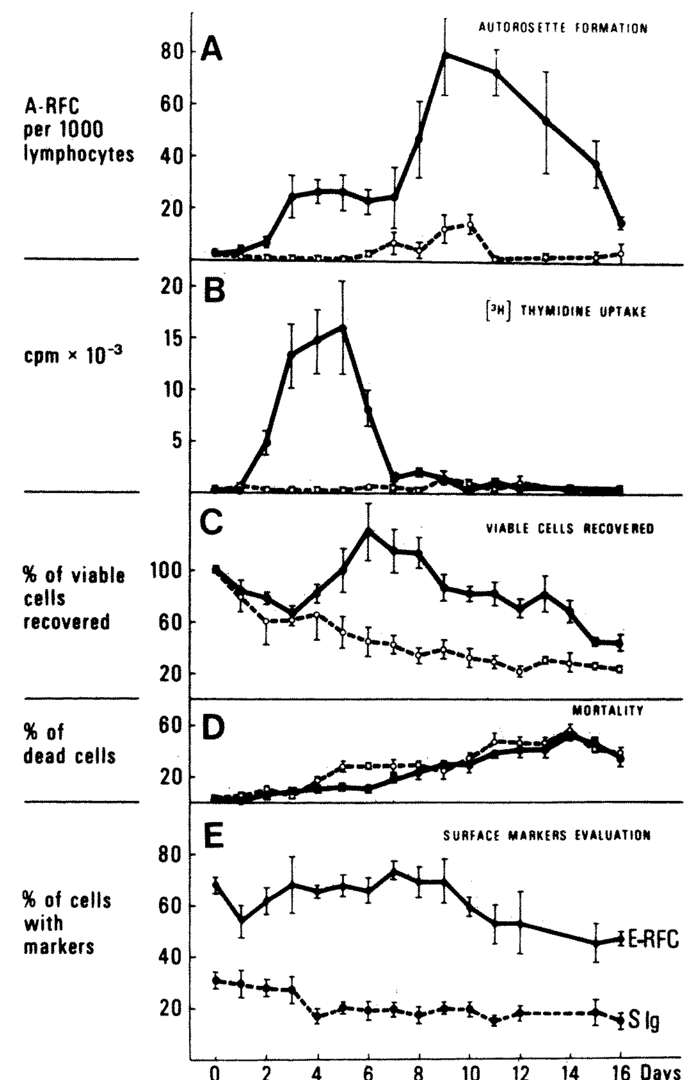

Figure 3. Time course of the proliferation response and the evolution of T and B markers in cultures stimulated with 2 μg/ml Con A (●——●) or unstimulated (○-----○). Mean ± S. E. of 7 to 14 determinations. *A*, autorosette formation; *B*, thymidine uptake (cpm × 10^{-3}); *C*, percentages of viable cells recovered after culture related to the original cell numbers; *D*, percentages of dead cells present in the suspensions; *E*, surface markers evaluation (◆——◆), E rosettes; (●-----●), sIg (surface immunoglobulin bearing cells) after Con A stimulation (unstimulated cultures are not shown).

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TABLE IV

Decrease in Con A-activated autorosettes after A-RFC depletion of initial suspensions at day 0, observed at 5 and 12 days after mitogen stimulation[a]

Expt. No.	Day 0: A-RFC per 1000 lymphocytes			Day 5: A-RFC per 1000 lymphocytes			Day 12: A-RFC per 1000 lymphocytes		
	Control cells	Depleted cells	% depletion	Control cells	Depleted cells	% depletion	Control cells	Depleted cells	Control cells
1	1.2	0.04	96	19.5	2.6	86	88.7	65.8	26
2	3.3	0.06	98	9.3	3.7	60	193.3	121.7	37
3	3.5	0.41	88				138.4	42.5	69
4	5.0	0.52	89	232.0	59.0	74	107.4	32.2	70
5	2.6	0.12	95	61.0	23.5	61	59.5	14.1	76

[a] PBL were obtained from 45- to 60-year-old female donors. All assays were performed at A-RBC:WC ratio = 64:1.

out of seven experiments). As can be seen (Table IV), a decrease in A-RFC levels was observed on day 5 and day 12. However, the depletion on day 5 was more pronounced than on day 12: 70% for 5 days *vs* 50% for 12 days (averages).

DISCUSSION

The present investigation confirms that a lymphocyte subpopulation of human peripheral blood, thymus, tonsils, lymph nodes, and spleen binds untreated autologous erythrocytes. These autologous red blood cell-binding lymphocytes appear to belong to a T cell subset composed of immature T cells: low autorosette levels are found in lymphoid organs containing a majority of mature T cells, such as lymph nodes or peripheral blood, whereas in the thymus where mostly immature T cells are present, high rosette values are obtained. The binding of autologous erythrocytes reaches 300/1000 thymocytes, whereas in the peripheral blood the level is usually 1 to 10 per thousand and never exceeds 30 per thousand cells even in aged women. In the spleen, lymph nodes, and tonsils, intermediate rates are observed. Similar results were found by Kaplan (4) in humans and by us in mice (15). A second argument for the immature nature of A-RFC is provided by the strong inhibition of autorosette levels after incubation *in vitro* with thymic extracts. The involvement of thymic factor in the expression of self-recognition was suggested by the increase in autologous rosettes with age concomitant with a decrease of serum thymic factor in humans (16) as well as in mice (17). Our findings are in agreement with the hypothesis that T cell maturation is associated with a loss in the expression of self-recognition receptors (18). These data are also in keeping with the absence of circulating thymic hormone in the serum of adult NZB mice (19) and human patients with systemic lupus erythematosus (SLE) (20). Since A-RFC appear to be immature T cells, it seemed of interest to activate them by Con A, a lectin that produces a stimulation of both mature and less mature T cell subsets (12–14). Moreover, it has recently been demonstrated that Con A activation of T cells can induce cytotoxicity against allogeneic and syngeneic targets (7–9). This phenomenon occurs after sensitization *in vivo* as well as *in vitro*. We have shown that the best Con A concentration to obtain an optimal stimulation of autorosettes was 2 μg/ml, a dose that is very low compared to that used for the proliferative response. Indeed, higher concentrations induced erythrocyte agglutination preventing rosette evaluation. Our results show that autorosette levels are strongly increased when PBL are stimulated with Con A. From day 1 to day 16 of culture we observed two peaks in the response: the first, appearing on day 3, represents a 10- to 15-fold stimulation of baseline autorosette levels. The second peak is much higher with a 30-fold increase in A-RFC numbers. This augmentation is not due to a nonspecific rosette-like agglutination by Con A since addition of Con A to lymphocytes and erythrocytes does not produce a similar increase in A-RFC values. It could be suggested that lymphoblasts produce autorosette formation by a nonspecific adherence of RBC. However, there was no correlation between the high incidence of A-RFC values found on days 9 to 11 and the proliferative response as measured either by evaluation of blasts on stained smears or by thymidine incorporation, which was diminishing at that time (Fig. 3*B*). Moreover, when Con A activation was competitively blocked by addition of α-methyl-D-mannopyranoside before rosetting, no decrease in autorosette numbers was noted. These data indicate that only a minority of the autorosettes formed with Con A-activated lymphocytes may reflect artifactual binding of erythrocytes to surface-bound Con A molecules, and therefore, it does not appear to be a significant factor. After stimulation with Con A, appearance of autorosettes coincides with the peak of thymidine incorporation and the consequent increase in cell numbers (Fig. 3), but the maximum was observed 5 or 6 days later. These data suggest that, even if most T cells transform and synthesize new DNA by day 3 or 4 in response to Con A (21, 22), A-RFC are probably generated through a process independent of DNA synthesis.

Autorosette formation also seems to be independent of the number of T or B cells, determined by surface marker characterization, since blast-transformed cells bore identical markers to initial small lymphocytes (23, 24). It could be postulated that the Con A-activated lymphocytes that bind autologous erythrocytes are similar to the cluster-forming cells (CFC) giving "giant rosettes" with sheep red blood cells (25, 26) or the "stable" E rosette-forming cells described by several investigators (27, 28). This view is based on the observation that all of these types of rosettes are common to mitogen-stimulated cells, but not to inactivated cells. In this regard, we have previously shown that autorosette-forming cells in human PBL can also bind sheep erythrocytes (6). Further, giant rosettes are inhibited by autologous red cells (25). Some notable differences exist, however, in the conditions required for the demonstration of the two types of rosettes. First, whereas a temperature of 37°C seems to be crucial for the demonstration of CFC, autorosette formation occurs after overnight incubation at 4°C, under conditions where no CFC are observed (25). Second, the kinetics and the levels of both types of rosettes are different: two peaks of giant rosettes are very sharp and are seen on days 3 and 7 after stimulation. At day 11 no CFC remain in the cultures. In contrast, our data indicate that the first peak of autorosettes forms a plateau from day 3 to day 6; then a further

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augmentation until day 13 is observed. Third, the Con A concentration used for the stimulation is 5 times lower in our culture conditions. Finally, it cannot be excluded that both A-RFC and CFC belong to the same T cell subset or are two overlapping T cell subpopulations. However, if this is the case, two distinct receptors for various types of red cells are present on the same lymphocyte and are visualized under different conditions, as suggested by Abuaf and Daguillard (25).

The evidence that Con A-stimulated autorosettes are produced by the cells that form spontaneous rosettes with autologous erythrocytes is supported by our depletion experiments: when initial cell suspensions were depleted of autorosettes and then cultured with Con A there was a decrease of 70% of these autorosettes on day 5. This result clearly demonstrates that Con A stimulates a clone of lymphocytes bearing receptors for autologous red blood cells. However, this phenomenon is less clear-cut for the second autorosette peak, on day 12, when only 50% depletion was observed. This could be accounted for by the A-RFC persisting at low levels in depleted suspensions, being sufficiently expanded by mitogen stimulation in culture by day 12 to increase the background level of A-RFC. This last possibility is confirmed by the fact that in unstimulated control cultures a slight increase in autorosettes was also noted at day 12.

It has been demonstrated in assays for helper function or induction of mitogen and allogeneic responses that Con A induces suppressor cells in mice and in men (29–36). Con A-stimulated autorosette-forming cells could belong to this suppressive population. However, the suppressive activity is usually described with higher Con A concentrations than ours and it appears during the first 24 to 48 hr of culture (32, 33). In conclusion, although Con A stimulation produces polyclonal activation against heteroantigens in some systems, our observations indicate that, in terms of A-RFC marker, Con A stimulation of human lymphocytes may also result in expansion of an autoreactive T cell population.

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