Clin. exp. Immunol. (1978) 32, 324–332.

Receptors for IgM: a feature of subpopulations of both T and B human lymphocytes

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(*Received* 28 *August* 1977)

SUMMARY

Receptors for IgM were detected on peripheral blood and tonsil human lymphocytes by a rosette technique with ox red blood cells (ORBC) coated with anti-ORBC rabbit IgM. It was found that the receptors are very sensitive to handling procedures of cells and to low temperatures.

An overnight incubation period at 37°C was the optimal condition for the maximum expression of receptors for IgM, but the use of IgM-free media in these cultures was neither an essential nor favourable factor for an optimal rosette formation, when ORBC heavily coated with rabbit IgM were used.

The great majority of chronic lymphocytic leukaemia (CLL) patients presented a high number of EA(IgM)-RFC, either on freshly drawn or cultured lymphocytes.

By fractionation procedures of normal peripheral blood and tonsil lymphocytes, it was found that a subpopulation of B cells, like T cells, also possess receptors for IgM. The receptors for IgM on B cells are less easily detectable and seem to possess a lower avidity for IgM than those present on T lymphocytes.

INTRODUCTION

It has been recently shown that human T-cell subpopulations can be identified by surface receptors capable of binding either the Fc portion of IgG or the Fc of IgM (Moretta *et al.*, 1975, 1976).

In the present study we have examined the ability of unfractionated and purified lymphocytes from the normal human peripheral blood and tonsil, and of chronic lymphocytic leukaemia (CLL) cells, to form rosettes with ox red blood cells (ORBC) coated with IgM antibody.

MATERIALS AND METHODS

Preparation of antisera against ORBC. A rabbit antiserum against ORBC containing mainly IgM antibody was raised in rabbits according to Mayer (1961). The antiserum was heated at 56°C for 30 min and subjected to Sephadex G-200 gel filtration. The antibody activity, as determined by complement-dependent haemolysis of ORBC, was virtually confined to the IgM-containing exclusion peak (titre: 1:2000). The mid-portion of this peak was collected and divided in small aliquots, which were stored at −20°C.

An antiserum against ORBC containing high titres of anti-ORBC IgG antibody was raised in rabbits by multiple intravenous injections of intact washed ORBC. The IgG fraction was purified by DEAE–cellulose chromatography. Both antisera were absorbed with human red cells prior to purification.

The purity of the IgM and IgG fractions was checked by the haemagglutination procedure and by the study of the ability of these fractions to react with staphylococcal protein A (SPA). For this purpose, *Staphylococcus aureus* strain Cowan I (National Collection of Type Cultures, London) and protein A–Sepharose CL-4B (Pharmacia, Uppsala) were used.

Preparation of lymphocytes and cell separation techniques. Tonsil and peripheral blood separated and unseparated lympho-

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cytes were prepared as previously reported (Romagnani *et al.*, 1977). Briefly, tonsil cells obtained by centrifugation at 400 *g* on a Ficoll–Hypaque gradient, and blood cells obtained by centrifugation on a Ficoll–Hypaque gradient followed by the depletion of phagocytes by magnetism, were fractionated by E rosetting for 1 hr at 4°C with neuraminidase-treated SRBC. E-RFC were separated from non-E-RFC on a Ficoll–Hypaque density gradient. The E-RFC were resuspended and centrifuged again on another density gradient. These procedures gave a suspension consistently containing more than 95% E-RFC and less than 2% Ig-bearing lymphocytes. SRBC were lysed by treatment with 0·87% NH_4Cl. The B-cell rich interface population was re-rosetted with neuraminidase-treated SRBC for 1 hr at 4°C and centrifuged on another Ficoll–Hypaque density gradient. This double-cycle procedure performed on tonsil cell suspensions gave populations contaminated by less than 1% E-RFC, whereas when peripheral blood was fractionated the number of contaminating E-RFC ranged between 2 and 7·5%.

Detection of lymphocyte surface markers. E-RFC and Ig-bearing lymphocytes were detected by techniques previously reported (Romagnani *et al.*, 1977).

To detect cells forming rosettes with ORBC coated with IgG (EA(IgG)-RFC), washed ORBC (4×10^8 per ml) were incubated for 30 min at room temperature with an equal volume of IgG anti-ORBC solution at a concentration of 200 μg/ml. After three washings ORBC were re-suspended at a concentration of 10^8 per ml and 0·1 ml of this suspension were added to 0·1 ml of the lymphocyte suspension (3×10^6 per ml). The mixture was centrifuged at 200 *g* for 5 min at 4°C. The pellet was gently resuspended and EA(IgG)-RFC counted immediately in a haemocytometer.

To detect cells forming rosettes with ORBC coated with IgM (EA(IgM)-RFC), washed ORBC (4×10^8 per ml) were incubated for 30 min at room temperature with an equal volume of IgM anti-ORBC solution at concentrations ranging between 10 and 320 μg/ml. After three washings, ORBC were resuspended in medium 199 at a concentration of 10^8 per ml and 0·1 ml of this suspension were mixed with 0·1 ml of the lymphocyte suspension (3×10^6 per ml) in 60×10 mm plastic tubes.

Either freshly drawn or cultured lymphocytes were tested. Cultured cells were kept at a concentration of 10^6 per ml in 5% CO_2–95% air at 37°C in medium 199 supplemented with heat-inactivated ORBC-absorbed 20% foetal calf serum (FCS). On occasions, parallel cultures containing 20% heat-inactivated ORBC-absorbed human AB or autologous serum were also performed.

The lymphocyte-ORBC mixture was centrifuged for 5 min at 200 *g* at 4°C and incubated for at least 30 min at 4°C. The pellet was gently resuspended and EA(IgM)-RFC scored in a haemocytometer.

Detection of EA(IgM)- and EA(IgG)-RFC after pronase treatment of lymphocytes. Peripheral blood lymphocytes cultured overnight in 20% FCS-containing medium were washed twice, adjusted at the concentration of 10^7 cells per ml in medium and incubated for 30 min at 37°C in the presence of different concentrations of pronase (Serva, Heidelberg). After three washings with medium containing 20% FCS the cells were resuspended in the same medium and tested for their capacity of forming EA(IgM)- and EA(IgG)-RFC.

Inhibition of EA(IgM)-RFC. Human IgM was prepared from pooled sera of patients with macroglobulinaemia by repeated dilution with distilled water followed by gel filtration on Sephadex G-200. Human IgG was prepared from pooled sera of normal individuals by precipitation with 33% saturated $(NH_4)_2SO_4$ followed by chromatography on a DEAE–cellulose column. The purity of IgM and IgG fractions was checked by immunodiffusion, immunoelectrophoresis and inhibition of haemagglutination. The protein concentration of purified IgM and IgG fractions was determined by the method of Lowry *et al.* (1951). IgG was aggregated by heating for 20 min at 63°C.

For the inhibition experiments, 0·05 ml of the lymphocyte suspension (6×10^6 per ml) obtained after overnight incubation at 37°C were mixed with 0·05 ml of the appropriate concentration of IgM or IgG solution. After a 1–2 hr incubation at 4°C, 0·1 ml of the ORBC suspension sensitized with rabbit IgM (160 μg/ml) were added to the mixture which was incubated again for 30 min at 4°C before reading.

RESULTS

Purity of sensitizing IgM solution

Since the use of an anti-ORBC IgM solution free of anti-ORBC-contaminating IgG was critical in this study, the purity of the sensitizing IgM antibody was checked by different procedures.

Tannic acid-treated SRBC sensitized with rabbita nti-ORBC IgG solution formed rosettes with fluorescent SPA-containing staphylococci, whereas SRBC sensitized with rabbit IgM did not. IgG-coated, but not IgM-coated, ORBC were capable of adhering to the surface of SPA–Sepharose beads. In addition, when the sensitizing capacity of the IgM solution (160 μg/ml) was evaluated before and after the elution on a column filled with SPA–sepharose beads, no significant differences were shown. On the contrary, the activity of the IgG solution (200 μg/ml) was completely abolished after elution through the SPA–sepharose column (Table 1).

Further evidence on the purity of the IgM solution was given by experiments of enzymatic treatment of lymphocytes. Concentrations of pronase as low as 10 μg/10^7 cells per ml were capable of inhibiting at all EA(IgM)-RFC, whereas the percentage of EA(IgG)-RFC was not significantly changed by treatment with pronase concentrations as high as 250 μg/10^7 cells per ml (Table 2).

TABLE 1. Effect of elution through a SPA–Sepharose column on the sensitizing capacity of the anti-ORBC IgM and IgG solutions*

	EA(IgM)-RFC (%)		EA(IgG)-RFC (%)	
Experiment number	HSA–Sepharose	SPA–Sepharose	HSA–Sepharose	SPA–Sepharose
(1)	25	30	28	< 1
(2)	22	28	18	< 1
(3)	23	19	25	< 1
(4)	25	26	26	< 1

* IgM (160 μg/ml) and IgG (200 μg/ml) solutions were eluted through a column of Sepharose beads activated by CNBr and conjugated with human serum albumin (HSA–Sepharose) or staphylococcal protein A (SPA–Sepharose). Rosetting was performed on peripheral blood lymphocytes cultured overnight in 20% FCS-containing medium.

TABLE 2. Effect of pronase treatment on the ability of lymphocytes to form EA(IgM) and EA(IgG) rosettes (results are percentages EA-RFC)*

		Pronase (μg/ml per 10^7 cells)			
Experiment number	Immunoglobulin	0	10	50	250
(1)	IgG	19	15	12	18
	IgM	40	< 1	< 1	< 1
(2)	IgG	22	23	22	16
	IgM	26	< 1	< 1	< 1
(3)	IgG	7	6	7	8
	IgM	65	< 1	< 1	< 1
(4)	IgG	15	19	17	18
	IgM	57	< 1	< 1	< 1

* In experiments (1) and (2) unfractionated cells were treated with pronase; in experiments (3) and (4) the pronase treatment was carried out on T cell-enriched populations. In all cases the cells were incubated overnight at 37°C, treated with pronase for 30 min and then tested with IgM- or IgG-sensitized ORBC.

Detection of EA(IgM)-RFC in freshly drawn and cultured human lymphocytes

In these experiments, the percentage of EA(IgM)-RFC was determined on peripheral blood lymphocytes (PBL) freshly drawn from twenty-three normal individuals and on the cells of the same donors cultured for 20 hr at 37°C in medium containing 20% FCS using ORBC heavily coated with anti-ORBC rabbit IgM (160 μg/ml). After a 20 hr incubation, the mean percentage of EA(IgM)-RFC was 33·9% whereas the percentage of EA(IgM)-RFC on the same blood populations, determined immediately after the end of separation procedures, was 12·4% (Table 3).

To establish whether or not the reduced number of EA(IgM)-RFC found in freshly drawn lymphocytes was due to the saturation of receptors with autologous IgM, the cells (10^6 per ml) were incubated for 20 hr at 37°C, either in 20% FCS- or 20% human AB pooled serum-containing media. The results of these experiments are shown in Table 4. High percentages of EA(IgM)-RFC could be demonstrated either in the FCS- or human AB serum-containing media. Thus it could be suggested that when ORBC, heavily coated with rabbit IgM, are used in the test the uptake of antigen–antibody complexes by the cells is not inhibited by the previous presence in the culture of autologous IgM, and that the variable reduction of the number of EA(IgM)-RFC found in freshly drawn lymphocytes could be related to the handling of the cells during the separation procedures rather than to the saturation processes.

TABLE 3. Percentage of EA(IgM)-RFC determined in PBL suspensions before and after culturing for 20 hr in FCS-containing media

Number of experiments	EA(IgM)-RFC (%) Before incubation	After incubation
23	12·4± 1·6 (range: 1·5–24)	33·9± 1·8 (range: 20–50)

The results indicate mean values ± s.e.m.

TABLE 4. Percentage of EA(IgM)-RFC determined in PBL suspensions cultured for 20 hr in media containing 20% FCS or AB human serum

Experiment number	EA(IgM)-RFC (%)		
	FCS	AB human serum	Autologous serum
(1)	46	40	—
(2)	29	33	—
(3)	24·5	32	—
(4)	24	17	—
(5)	21	36	—
(6)	34	65	55
(7)	41	62	62
(8)	49	65	56
Mean value ± s.e.m.	33·5 ± 3·8	45 ± 7	—

To verify this hypothesis, PBL were tested for their ability to form EA(IgM)-rosettes immediately after the end of the separation procedures and after 2 and 4 hr of incubation at 4°C and 37°C in FCS-containing medium. As shown in Fig. 1, the percentage of EA(IgM)-RFC present at time 0 was increased by incubation at 37°C for 2 hr and was increased much more after a 4 hr incubation, whereas it remained at a low level if incubated at 4°C.

Moreover, cord blood lymphocytes, which one can assume to possess receptors unsaturated by autologous IgM, showed the same behaviour as of adult lymphocytes. In fact, the number of EA(IgM)-RFC found immediately after the end of the separation procedures was much higher than that demonstrable after a further 6 hr incubation at 37°C in FCS-containing medium (Fig. 2). Finally, the number of EA(IgM)-RFC found in cell populations cultured overnight at 37°C in serum-containing medium was significantly decreased after the centrifugation of cultured cells on a Ficoll–Hypaque gradient and three washings. The mean value of the decrease was greater than 50% (Table 5).

These results show that the receptors of handled cells are very sensitive to the influence of time and temperature. However, when the cells cultured for 20 hr at 37°C in 20% FCS were centrifuged, resuspended in 60% FCS or AB human serum and incubated again for 1–3 hr at 4°C before testing for their ability to form EA(IgM) rosettes, a significant reduction in the number of EA(IgM)-RFC in AB serum-incubated in comparison to FCS-incubated suspensions was seen (Table 6). These data strongly suggest that high concentrations of human AB serum can inhibit, at 4°C, the attachment of ORBC coated with rabbit IgM to lymphocytes.

Detection of EA(IgM)-RFC in PBL suspensions of patients with CLL

When PBL suspensions from patients with CLL were tested for their ability to form EA(IgM)

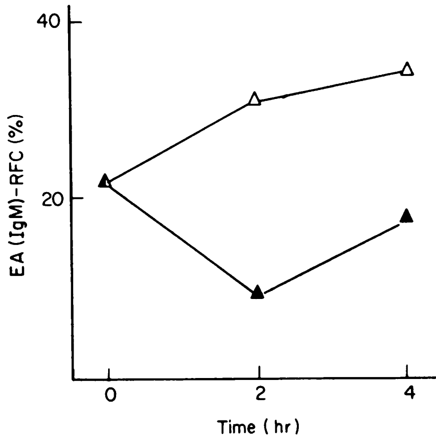

FIG. 1.

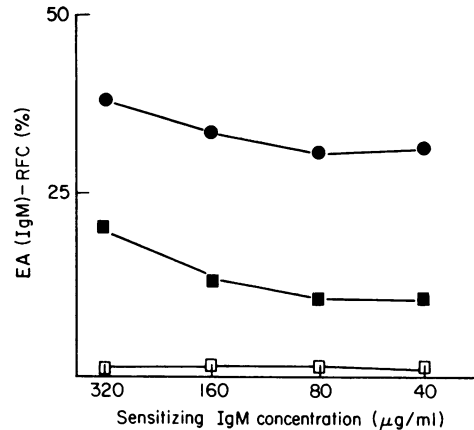

FIG. 2.

FIG. 1. Effects of time and temperature on the receptors for IgM on PBL. EA(IgM)-RFC were detected immediately after the end of isolation procedures (time 0) and after 2 and 4 hr incubations at 4°C (▲) and 37°C (△) in 20% FCS-containing medium. The mean values of four separate experiments are reported.

FIG. 2. Effects of the time and temperature on the receptors for IgM on cord blood lymphocytes. EA(IgM)-RFC were detected immediately after the end of isolation procedures (■), after a 2 hr incubation at 4°C (□) and a further 6 hr incubation at 37°C (●) in 20% FCS-containing medium. Several concentrations of sensitizing rabbit IgM were used. The mean values of four separate experiments are reported.

TABLE 5. Effect of centrifugation on a Ficoll–Hypaque gradient and three washings on the ability of lymphocytes cultured overnight at 37°C in 20% FCS to form EA(IgM) rosettes

	EA(IgM)-RFC (%)	
Experiment number	Before*	After†
(1)	41·5	20
(2)	17	2
(3)	36	14
(4)	51	34·5

* EA(IgM)-RFC were detected on cell suspensions cultured overnight at 37°C in 20% FCS-containing medium.

† The tests were performed on the same cultured cells after centrifugation on a Ficoll–Hypaque gradient and three washings with medium.

rosettes, a very high proportion of EA(IgM)-RFC was seen, either in freshly drawn or cultured suspensions of cells from the great majority of patients with CLL (Table 7).

Detection of EA(IgM)-RFC in purified cell populations of tonsils and peripheral blood

Purified T and B lymphocytes from human tonsils cultured for 20 hr at 37°C in FCS-containing medium were tested for their ability to form EA(IgM) rosettes by using ORBC coated with anti-ORBC rabbit IgM at concentrations ranging between 10 and 320 μg/ml. As reported in Fig. 3, high percentages of EA(IgM)-RFC could be found in purified T-cell populations with almost all the IgM-coated ORBC

TABLE 6. Inhibition of EA(IgM)-RFC by a 2 hr incubation at 4°C of PBL cultured for 20 hr at 37°C in FCS-containing medium

Cell donors	EA(IgM)-RFC (%) after 2 hr incubation in:		
	FCS (at 4°C)	AB human serum	
		At 4°C	At 37°C
Normal	38	16	42
Normal	39	5	43
Normal	35	8	41
CLL	50	33	43
CLL	25	14	40

TABLE 7. Percentage of EA(IgM)-RFC and EA(IgG)-RFC determined in freshly drawn PBL suspensions of patients with CLL

Patient number	E-RFC	Ig-bearing cells	EA(IgG)-RFC	EA(IgM)-RFC
(1)	5	90	45	92
(2)	20	72	32	89
(3)	20	80	11	65
(4)	16	83	3	14
(5)	14	85	79	42
(6)	4	95	50	52
(7)	4	93	17	58
(8)	7	84	—	60
(9)	18	80	74	76
(10)	10	90	41	45

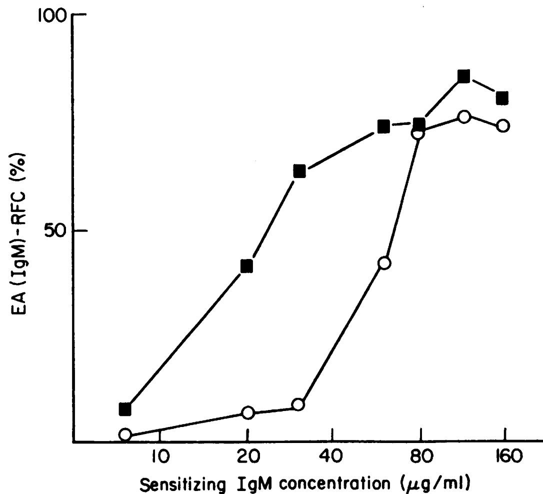

FIG. 3. Percentage of EA(IgM)-RFC detected on B cell-enriched (○) and B cell-depleted (■) tonsil populations by ORBC coated with different concentrations of rabbit IgM. The results are expressed as the percentages of EA (IgM)-RFC found by using ORBC with the maximum rabbit IgM concentration (320 μg/ml). The mean values of three experiments are reported.

samples, whereas only ORBC coated with high concentrations of IgM were able to form EA(IgM) rosettes with purified B-cell populations.

The detection of EA(IgM)-RFC on B cell-enriched populations from the peripheral blood presented greater technical difficulties. After incubation for 20 hr in FCS-containing medium, peripheral blood B cell-enriched suspensions did not constantly form a significantly greater number of EA(IgM) rosettes than that which could be expected on the basis of the number of contaminating E-RFC. However, these populations showed a significantly greater number of EA(IgM)-RFC after incubation for 20 hr at 37°C in the AB serum-containing medium (Fig. 4).

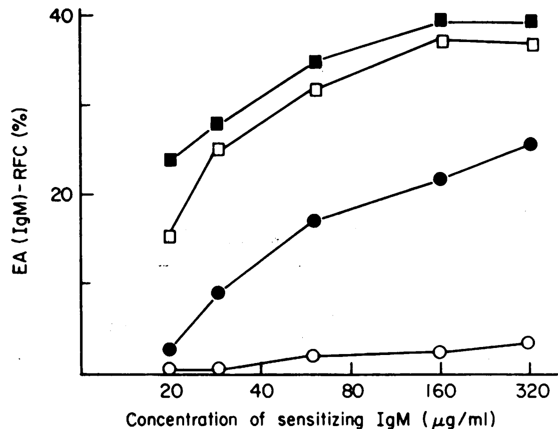

FIG. 4. Percentage of EA (IgM)-RFC on B cell-depleted (■, □) and B cell-enriched (●, ○) populations from peripheral blood cultured overnight in 20% FCS- (□, ○) or 20% AB human serum (■, ●) containing medium. The mean values of four separate experiments are reported.

Inhibition with human IgM or IgG of EA(IgM)-RFC

In these experiments, purified T and B cells from human tonsils cultured for 20 hr in 20% FCS-containing medium were further incubated for 1 hr at 4°C with various concentrations of human IgM or IgG before testing with IgM-coated ORBC. A greater than 50% inhibition of EA(IgM) rosettes formed by T cells was observed when concentrations as low as 40–80 μg/ml were used. On the contrary, to obtain a greater than 50% inhibition of EA(IgM) rosettes formed by the B cells, IgM concentrations equal or greater than 600 μg/ml should be used (Fig. 5). A similar IgM concentration was also necessary to significantly inhibit the formation of EA(IgM) rosettes by CLL cells. Either soluble or heat-aggregated human IgG, even at concentrations as high as 4 mg/ml, consistently failed to inhibit EA(IgM)-RFC. On the contrary, heat-aggregated IgG normally induced a more than 50% inhibition of EA(IgG)-RFC found in the T cell-depleted populations from the peripheral blood.

DISCUSSION

The results reported in this paper show that B cells, like T cells, are equipped with receptors for antigen–IgM antibody complexes. Previously, Moretta *et al.* (1975) demonstrated that IgM receptors can be detected on a noticeable proportion of human T lymphocytes using a rosette technique with ORBC lightly coated with rabbit IgM antibody. These receptors could not be detected on PBL freshly drawn from donors, but they were expressed by cells kept *in vitro* for 24 hr, particularly when cultured in media containing very little or no IgM. Similar results were also reported by McConnel & Hurd (1976). On the contrary, Gmelig-Meyling, van der Ham & Ballieux (1976) found that the advised overnight incubation period at 37°C to make the IgM receptor demonstrable was neither an essential nor a favourable factor for an optimal rosette formation.

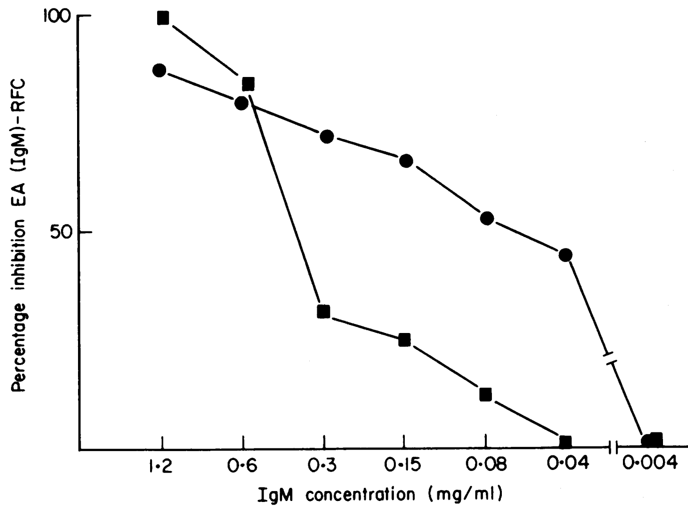

FIG. 5. Inhibition of EA(IgM)-rosettes formed by purified T (●) and B (■) tonsil lymphocytes by different concentrations of human IgM. 50 μl of the lymphocyte suspension ($0{\cdot}3 \times 10^6$ cells) were incubated with 50 μl of the appropriate concentration of human IgM for 1 hr at 4°C before adding the sensitized ORBC.

On the basis of our data, these controversial findings can be explained by the sensitivity of the IgM receptor to the variable handling to which the lymphocytes are subjected during the separation procedures. In particular, our experiments showed that the number of EA(IgM)-RFC found after centrifugation of blood cells on the Ficoll–Hypaque gradient and three washings was significantly lower than that found after a 6–24 hr incubation at 37°C, which could be considered the optimal condition for the maximum expression of receptor on the T cells. Moreover, the incubation of the cells with carbonyl iron on a rotator for the depletion of phagocytic cells, or rotation alone of the cells for the same length of time after centrifuging on the Ficoll–Hypaque gradient, resulted in a further decrease of the number of EA(IgM)-RFC. The decrease was much more evident if the cells at the end of these procedures were stored for 2 hr at 4°C. When EA(IgM)-RFC were checked on purified cell suspensions at the end of the fractionation procedures, which need a still more marked and prolonged handling of the cells, no EA-(IgM)-RFC could be detected. On the basis of these data, one can thus speculate that IgM receptors are easily shed, especially when the cells are washed and/or stored at 4°C. However, the incubation of the handled cells at 37°C for 4–6 hr is sufficient to induce the resynthesis of the receptors on a noticeable proportion of cells.

The use of IgM-free media during the incubation period at 37°C was neither an essential nor a favourable condition for the expression of these receptors. Probably the amount of IgM normally present in the autologous or homologous serum is not sufficient to mask the receptors of the cells incubated at 37°C, especially when ORBC heavily coated with rabbit IgM are used to detect the EA(IgM)-RFC.

Recently, it has been shown that a high percentage of freshly drawn PBL from CLL patients are able to form EA(IgM) rosettes. This finding was interpreted as a particular feature of the malignant proliferation of B cells (Pichler & Knapp, 1977). Our results also demonstrate that in the great majority of patients with CLL a considerable number of EA(IgM)-RFC can be detected on the PBL suspensions tested either before or after the incubation at 37°C. On the other hand, by fractionation experiments performed on the blood and tonsil populations from normal subjects, we could demonstrate that the expression of IgM receptors on CLL cells is not a unique feature of the malignant proliferation, since a noticeable proportion of normal B lymphocytes is also equipped with receptors for IgM.

Our data also show that receptors for IgM can be detected on B cells when ORBC coated with larger amounts of rabbit IgM than those sufficient to detect IgM receptors on T lymphocytes are used. Since B lymphocytes also possess receptors for IgG, one could speculate that the binding of ORBC coated with an IgM solution is due to contaminating IgG. However, this possibility should be excluded

by tests for purity of the reagents and by additional findings. The IgM solution was not able to react with SPA and its activity was not changed by the absorption on a SPA–sepharose column, which was proposed as a substitute for sera directed against IgG (Bächi *et al.*, 1977; Biberfeld, Ghetie & Sjöquist, 1975). On the other hand, the sensitizing capacity of the solution used by us to identify EA(IgM)-RFC, which contained a concentration of anti-ORBC IgG which was undoubtedly much greater than that supposed to contaminate the IgM solution, was completely inhibited by the absorption on the SPA–sepharose column. In addition, in the B cell-enriched populations from the tonsil, where we never did find more than 3% EA(IgG)-RFC, relatively high proportions of EA(IgM)-RFC could be detected. Finally, ORBC coated with the IgM solution were unable to form rosettes with lymphocytes treated with very low concentrations of pronase, whereas those coated with the IgG solution reacted well with the lymphocytes, even after they were treated with a more than twenty times greater concentration of pronase. These data are in agreement with the findings recently reported by Moretta *et al.* (1977), which showed a differing sensitivity of the receptors for IgM and IgG present on T lymphocytes to digestion with pronase.

Our data also show that EA(IgM)-RFC found on B cell-purified suspensions from the peripheral blood are more easily demonstrable when the cells are incubated overnight at 37°C in 20% AB human serum- instead of FCS-containing medium, probably because the incubation in homologous serum represents better conditions than incubation in heterologous serum. In addition, the inhibition experiments showed that a concentration of human IgM much greater than that which was sufficient to inhibit the EA(IgM) rosettes formed by the T cells was necessary to block the EA(IgM) rosettes formed by the B cells. Thus one can speculate that B cells are equipped with receptors for IgM possessing a lower avidity than those present on T lymphocytes.

This work was supported by funds from the 'Consiglio di Amministrazione dell'Università di Firenze, capitolo XI/b' and from the CNR (Contratto No. 7500684/04).

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