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Mouse rosette positive B cells stimulate poorly in the autologous and allogeneic mixed lymphocyte reaction

F. R. DAVEY & A. S. KUREC *Division of Clinical Pathology, State University of New York, Upstate Medical Center, Syracuse, New York, USA*

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SUMMARY

Mouse erythrocytes form spontaneous rosettes with a population of B lymphocytes from normal individuals and in the majority of lymphocytes from patients with B cell chronic lymphocytic leukaemia (CLL). We have compared the ability of mouse rosette positive (MR^+) cells with mouse rosette negative (MR^-) cells and monocytes to act as stimulators in the autologous mixed lymphocyte reaction (AMLR) and allogeneic mixed lymphocyte reaction (MLR). Mononuclear cells from the peripheral blood of healthy individuals were fractionated into T cells, MR^+ cells, MR^- cells and monocytes. Lymphocyte cultures were harvested on days 6, 8 and 10 and the incorporation of tritiated thymidine was determined. MR^- cells and monocytes were potent stimulators in the AMLR and MLR. In contrast MR^+ cells, like B cells from patients with CLL, stimulated less in the AMLR and MLR. We conclude that MR^+ cells from normal individuals function similarly to cells from CLL in the AMLR and MLR.

INTRODUCTION

A subpopulation of B lymphocytes from normal individuals form spontaneous rosettes with mouse erythrocytes (Stathopoulos & Elliott, 1974; Gupta & Grieco, 1975). These mouse rosette positive (MR^+) B cells are present early in ontogeny (Gupta *et al.*, 1976b) and are associated with cells bearing surface membrane immunoglobulins, IgM and IgD but not IgG (Gupta, Good & Siegal, 1976a). In addition, peripheral blood B lymphocytes lose their ability to form mouse rosettes following incubation with pokeweed mitogen (McGraw, Kurec & Davey, 1982). These data suggest that the mouse rosette receptor may represent a marker for early or resting B lymphocytes.

A variety of lymphoproliferative disorders have been investigated for the presence of MR^+ cells. In almost all cases of chronic lymphocytic leukaemia (CLL) (Catovsky, Wechsler & Cherchi, 1981) and approximately 40% of cases of hairy cell leukaemia (HCL) (Catovsky *et al.*, 1975; Burns & Cawley, 1980), MR^+ cells are increased in number in the peripheral blood. In some studies of patients with CLL, the mouse rosette assay is more consistently positive than the determination of surface membrane immunoglobulin (Cherchi & Catovsky, 1980). In contrast few other lymphoproliferative disorders exhibit increased number of MR^+ cells (Koziner *et al.*, 1977, 1980; Catovsky *et al.*, 1976).

Lymphocytes from patients with CLL (Wolos & Davey, 1980a) and HCL (Davey, Dock &

Correspondence: Professor F. R. Davey, Division of Clinical Pathology, State University of New York, Upstate Medical Center, 750 East Adams Street, Syracuse, New York 13210, USA.

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Wolos, 1980) stimulate poorly in the autologous mixed lymphocyte reaction (AMLR) and the allogeneic mixed lymphocyte reaction (MLR). Previous studies have indicated that this lack of stimulation is not the result of suppressor factors, suppressor cells or lack of responsive T cells (Wolos & Davey, 1979, 1980b). Therefore, the lack of a stimulatory signal by CLL lymphocytes may be the result of an intrinsic inability of CLL cells. It is possible that CLL is a neoplastic proliferation of B lymphocytes at a stage in the immunological maturation characterized by the presence of the mouse rosette receptor but lacking the concentration of antigens necessary for a vigorous stimulatory response in the AMLR and MLR. If this hypothesis is correct, then perhaps MR^+ cells from normal individuals may also stimulate poorly in the AMLR and MLR. The purpose of this report is to compare the stimulatory capacity of MR^+ cells, MR^- cells and monocytes from normal individuals in the AMLR and MLR.

MATERIALS AND METHODS

Cell source. Mononuclear cell suspensions were isolated by gradient centrifugation on lymphocyte separation medium (LSM, Bionetic, Kensington, Maryland, USA) from normal, healthy subjects or residual cells from plateletphoresis donors (Dock & Davey, 1980). The cells were washed three times with Hank's balanced salt solution (HBSS; GIBCO, Grand Island, New York, USA), and brought to a final concentration of 1×10^6/ml in HBSS.

Separation of mononuclear cell subpopulations

T cell enriched population. An equal volume of the mononuclear cell suspension was mixed with 1% 2-amino-ethyl-isothiouroniumbromide hydrobromide (0·14M AET, pH 9·0, Sigma Chemical Co., St Louis, Missouri, USA) treated sheep erythrocytes (Krutulis Laboratories, Bridgeport, New York) in 40% heat-inactivated fetal calf serum (FCS; GIBCO) and HBSS, in a 50 ml conical centrifuge tube. After centrifugation for 5 min at 200 ***g***, the pellet was gently resuspended and carefully underlayed with 10–15 ml of LSM, and again centrifuged for 30 min at 400 ***g***. The interface cell suspension (consisting of non-T cells; B cells, monocytes and null cells) was decanted, washed and resuspended in 20% FCS plus RPMI 1640 with 100 units/ml of penicillin, 50 μg/ml of streptomycin, and 0·3 mg/ml of L-glutamine (Associated Bionetics, Buffalo, New York) at a concentration of 20×10^6 cells/ml. The erythrocytes in the pellet were lysed with pre-warmed (37°C) 0·85% Tris–ammonium chloride (pH 6·3, Sigma Chemical Co.). The lymphocytes in the pellet were washed several times with HBSS and finally resuspended in HBSS with 10% FCS at a concentration of less than 50×10^6/ml. This cell suspension was then incubated in a prescubbed nylon wool (Associated Bionetics) column (Wolos & Davey, 1980a). The non-adherent cells eluted from this column were depleted of non-T cells and enriched for T lymphocytes.

B cell and monocyte enriched populations. The non-T cell population was depleted of monocytes by incubating the cell suspension on a 100×20 mm plastic culture dish (3003, Falcon, Oxnard, California, USA) for a minimum of 1 hr at 37°C in a 5% CO_2 humidified environment. Following incubation, the cells were removed by gentle rinsing with 20% FCS in RPMI 1640, the non-adherent cells were listed as the B and null cell population. The adherent cells were removed by incubating the cells with HBSS without Ca^{++} or Mg^{++} for 10 min at room temperature, and then gently scraped with a rubber policeman. The adherent cells were considered the monocyte enriched population.

MR^+ and MR^- enriched populations. The MR^+ population was obtained using a modification of the method by Dolan & Park (1978). The B and null cell suspension was resuspended in normal saline (0·9% NaCl) at a concentration of 4×10^6/ml. Fresh mouse erythrocytes (AJAX, Jackson Laboratory, Bar Harbor, Maine, USA) were treated with neuraminadase (0·2 ml of 1 units/ml of neuraminidase per 1 ml of a 5% suspension of washed mouse erythrocytes; GIBCO) for 1 hr at 37°C. The treated mouse red blood cells (MRBC-N) were washed and resuspended in 40% FCS and 0·9% NaCl to a concentration of $0{\cdot}14 \times 10^9$/ml. Equal volumes of B, null cells and MRBC-N were mixed, incubated for 15 min at 37°C, and centrifuged at 200 ***g*** for 5 min. The pellet was left undisturbed overnight at 4°C. In the morning, the pellet was gently resuspended and underlayed with LSM. The interface population was washed with HBSS and labelled as MR^- cells. The erythrocyte pellet was

lysed with Tris–ammonium chloride and washed with HBSS and considered to be the MR^+ cell population.

Mononuclear cell markers. Each subpopulation was tested for sheep erythrocyte rosette forming cells (Wybran, Carr & Fudenberg, 1972), Pan-T cell antigen (Leu 1) (Wang *et al.*, 1980), surface membrane immunoglobulin (Papamichail, Brown & Holborow, 1971), Fc receptor (Dickler & Kunkel, 1972), Ia antigen (Davey *et al.*, 1980), alpha-naphthyl acetate esterase (Yam, Li & Crosby, 1971), and colony forming unit cultures (CFUc) (Goldberg *et al.*, 1980). The cellular composition and viability of each subpopulation are given in Table 1.

Mixed lymphocyte cultures. Each cell population was resuspended in culture media (20% FCS in RPMI 1640 plus 3 ml 1M HEPES Buffer, pH 8·1; GIBCO) at a concentration of 1×10^6/ml. A

Table 1. Cell markers of enriched mononuclear cell populations

	Mean percentage positive ± s.e.m.						Mean %±s.e.m.	Mean No./5×10^5† cells plated
	ER*	Leu 1*	SMIg*	Fc*	Ia*	αNAE*	Viable	CFU-C
T cells	66±17	66±18	1±1	2±2	9±6	1±1	89±7	—
MR^+ cells	2±3	2±3	58±12	39±18	78±13	11±12	85±5	7
MR^- cells	5±9	1±1	33±11	50±20	78±15	23±14	89±8	81
Monocytes	2±3	<1	28±16	54±15	73±19	80±14	86±6	—

* ER = spontaneous rosette forming cells with sheep erythrocytes; Leu-1 = Pan T cell antigen; SMIg = Surface membrane immunoglobulin; Fc = Fc receptor (for IgG); Ia = Ia-like antigen; αNAE = alpha-naphthyl acetate esterase (diffuse reaction).

† CFU-c = Number of clusters on soft agar cultures at day 14.

predetermined number of the stimulating cell population was treated with 2 ml of mitomycin-C (0·025 mg/ml; ICN Pharmaceuticals, Cleveland, Ohio, USA) for 30 min at 37°C. The cells were washed three times and resuspended to 1×10^6/ml in culture media. Using flat bottom tissue culture plates (Costar, Rochester Scientific, Rochester, New York), 1×10^5 responding cells were added to 1×10^5 mitomycin-C treated stimulating cells in a total volume of 200 μl of culture media. These cultures were incubated at 37°C in a 5% CO_2 atmosphere and harvested on days 6, 8 and 10. Cultures were pulsed with 25 μl of 1 μCi of tritiated thymidine (Amersham, Arlington Heights, Illinois, USA) per well. Twenty-four hours later cultures were harvested with a multiple automatic sample harvestor (MASH; Brandel, Gaithersberg, Maryland, USA) onto glass fibre discs, dried, and counted in a beta scintillation coulter.

Statistical analysis. When appropriate, the data were compared using a Student's paired *t*-test or the Dunnett's procedure according to Steel & Torrie (1960).

RESULTS

Autologous mixed lymphocyte reaction

Since previous studies in our laboratory indicated that the peak proliferative response in the AMLR occurred between the 8th and 10th culture days, we performed AMLR on lymphocytes collected from six individuals and measured the proliferative response on the 8th culture day (Table 2). The data demonstrated that MR^- cells and monocytes stimulate T cells well in the AMLR whereas MR^+ cells were relatively poor stimulators. The proliferative response of MR^+ was significantly less than that observed for MR^- cells ($P < 0{\cdot}02$). When monocytes were added to MR^+ and to MR^- cell populations in 1:1 proportions, the proliferative responses generated by these stimulatory cell populations were no longer significantly different.

To determine if the diminished stimulation observed with MR^+ cells was the result of a shift in kinetics of this cell population, four additional cultures were assayed for a proliferative response on three different days. The peak proliferative response generated by MR^+ cells was always less than that by MR^- cells and monocytes (Table 3).

Allogeneic mixed lymphocyte reaction

In six additional experiments, the proliferative response of allogeneic lymphocytes stimulated by MR^+ cells, MR^- cells and monocytes was also determined. On the 6th, 8th and 10th culture days, the proliferative response generated by the MR^+ cells was consistently less than that observed for MR^- cells and monocytes ($P < 0{\cdot}01$; Table 4). When monocytes were added to MR^+ and MR^- cell populations in 1:1 proportions, the proliferative response of the MR^+ cells increased to the level observed with MR^- cells plus monocytes.

DISCUSSION

The data from the current study indicated that MR^+ cells stimulated less than did MR^- cells and monocytes in the AMLR and MLR. The MR^+ cell population was enriched for B cells whereas the MR^- cell population was more heterogeneous than the MR^+ fraction but, nevertheless, enriched for null cells as demonstrated by the high number of CFC-c cells. Previous studies (Beale *et al.*, 1980; Kuntz, Innes & Weksler, 1976; MacDermott & Stacey, 1981) using similar and other methods to separate mononuclear cells have also demonstrated that monocytes and null cells were potent stimulators in the AMLR. However, in these studies, the B cell fraction usually generated a strong proliferative response in the MLR. In the current experiments, MR^+ cells consistently stimulated less in the MLR than did the MR^- cells and monocytes. It is possible that the MR^+ cell fraction was composed of a subpopulation of B cells with an inability to produce a strong stimulatory signal in the MLR as well as the AMLR. Previous studies (Gupta & Grieco, 1975) indicated that MR^+ cells are more associated with lymphocytes bearing IgM and IgD than those containing IgG surface immunoglobulin. It is possible that the latter B cells are more potent stimulators in the MLR. Several studies have demonstrated that the Ia-like antigens or HLA-DR antigens are necessary determinants in the stimulation of a proliferative response in the AMLR and MLR (Huber *et al.*, 1981; Bergholtz, Albrechtsen & Thorsby, 1977). Although the MR^+, MR^- and monocyte cell populations possessed a similar percentage of Ia^+ cells, it is possible that the concentration of Ia^+ like antigens per cell was less or perhaps qualitatively different in MR^+ cells than MR^- cells and monocytes. Since in the current study the addition of monocytes to the MR^+ cell population enhanced the stimulatory response, it is possible that monocytes either provided the predominant stimulatory signal or modulate the stimulatory response of the MR^+ population. Studies by Hausman *et al.* (1980) have indicated that the AMLR is a heterogeneous response and reflects the proliferation of two distinct T cell populations in response to distinct stimulator cells, a monocyte population and a B cell population. The latter group would include both our MR^+ and MR^- populations. We believe that the proliferative response stimulated by the B cell enriched fraction in the studies by Hausman *et al.* (1980) was owing to the inclusion of the MR^- population.

Mouse erythrocytes bind spontaneously to a subpopulation of B lymphocytes (Gupta & Grieco, 1975). Thymocytes, T cells, null cells, stimulated lymphocytes, plasma cells, monocytes and myeloid cells appear to be negative for the mouse rosette receptor (Gupta *et al.*, 1976a; Dolen & Park, 1978; McGraw *et al.*, 1982). The receptor for mouse rosettes may be associated with the early stages of B cell maturation because MR^+ cells are observed in fetal liver during the early stages of the ontogeny of B lymphocytes (Gupta *et al.*, 1976b). In addition, the proportion of MR^+ cells is higher in B cell populations when IgM is the main heavy chain but declines as other surface heavy chains appear (Koziner *et al.*, 1980).

MR^+ cells represent 25–90% of cells in virtually all cases of CLL (Catovsky *et al.*, 1981). In approximately 40% of cases of HCL, MR^+ cells represent greater than 25% of neoplastic cells (Catovsky *et al.*, 1975). Other lymphoid malignancies are usually negative for the mouse rosette receptor (Catovsky *et al.*, 1976; Koziner *et al.*, 1977, 1980).

Table 2. Autologous mixed lymphocyte reaction

Responding cell population	Stimulating cell* population	Mean NET† c.p.m. ± 1 s.e.m.
T cells	MR^+ cells	25,470 ± 5,981
T cells	MR^- cells	55,235 ± 4,250
T cells	Monocytes	39,023 ± 9,289
T cells	MR^+ cells + monocytes	49,109 ± 7,044
T cells	MR^- cells + monocytes	52,179 ± 5,138

* MR^+ = Mouse rosette positive cells; MR^- = Mouse rosette negative cells.

† MR^+ vs MR^- cells; $P < 0{\cdot}02$.

Table 3. Proliferative response of autologous mixed lymphocyte reaction (mean net c.p.m.)*

Days of culture	Composition of culture†		
	$T+MR^+_m$	$T+MR^-_m$	$T+M_m$
6	5,623 ± 3,300	15,138 ± 6,536	13,630 ± 3,312
8	13,846 ± 7,304	29,150 ± 11,612	31,884 ± 8,167
10	13,482 ± 8,165	21,950 ± 11,216	21,909 ± 3,866

* Mean of four experiments ± 1 s.e.m.

† T = autologous T responding cells; subscript m = mitomycin-C treated stimulating cells; MR^+ = mouse rosette positive cells; MR^- = mouse rosette negative cells; M = monocytes.

Table 4. Proliferative response of allogeneic mixed lymphocyte reaction (mean net c.p.m.)*

Days of culture	Composition of cultures†					
	$A+B_m$	$A+MR^+_m$‡	$A+MR^-_m$	$A+M_m$	$A+(MR^++M)_m$	$A+(MR^-+M)_m$
6	31,733	8,799	27,690	49,235	28,506	29,415
	± 4,492	± 5,192	± 6,319	± 9,158	± 7,497	± 6,385
8	60,615	8,749	56,150	61,451	44,038	48,780
	± 13,745	± 5,567	± 15,305	± 11,908	± 12,715	± 11,951
10	20,224	6,783	32,167	27,961	28,852	26,619
	± 10,541	± 3,959	± 10,962	± 6,202	± 7,543	± 9,463

* Mean of six experiments ± 1 s.e.m.

† A = Allogeneic responding cell population; Subscript m = Mitomycin-C treated stimulating cell population; B = unfractionated lymphocytes; MR^+ = Mouse rosette positive cells; MR^- = Mouse rosette negative cells; M = monocytes.

‡ $A+MR^+_m$ vs $A+MR^-_m$ and $A+MR^+_m$ vs $A+M_m$; $P < 0{\cdot}01$.

Lymphocytes from patients with CLL stimulate poorly in the AMLR (Wolos & Davey, 1980a) and in the MLR (Wolos & Davey, 1981). In the MLR, the proliferative response generated by stimulating CLL cells was inversely proportional to the peripheral blood leucocyte count suggesting that the normal stimulatory cells were being diluted out by neoplastic non-stimulatory cells. Furthermore, previous studies (Wolos & Davey, 1979) showed that the lack of a proliferative response was not due to the presence of a suppressor cell or suppressor serum factor.

The current study and other reports (Catovsky *et al.*, 1976, 1981; Koziner *et al.*, 1980) indicate that MR^+ cells from normal individuals share several characteristics in common with neoplastic CLL cells. They are both small B lymphocytes (unpublished results; Chapman, Kurec & Davey, 1981) and possess SMIg, Ia-like antigens, Fc and C3 receptors (Monhanakumar *et al.*, 1979, Bertoglio *et al.*, 1977; Koziner *et al.*, 1980). In addition, the current study indicates that MR^+ cells from normal individuals function in the AMLR and MLR like lymphocytes from patients with CLL. Thus it remains possible that the mouse rosette receptor is a marker for the early stages of B cell maturation and that these B cells accumulate excessively in the peripheral blood, bone marrow and tissues of patients with CLL.

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