Journal of Chromatography, 376 (1986) 401—408
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2963

SEPARATION OF HUMAN LYMPHOID CELLS BY AFFINITY CHROMATOGRAPHY AND CELL SURFACE LABELLING BY HYDROXYETHYL METHACRYLATE PARTICLES USING MONOCLONAL ANTIBODIES

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SUMMARY

The hydroxyalkyl methacrylate gel Separon, Sepharose 6MB and polystyrene dishes with bound antibodies were used for affinity chromatography of cells isolated from human tonsils and peripheral blood. Enrichment of T cells by filtration through anti-human immunoglobulin Separon or Sepharose 6MB columns was comparable to that achieved by "panning" on dishes or by Nylon wool column fractionation. High enrichment of B cells was obtained when cells pretreated with monoclonal anti-T cell antibodies were fractionated on columns or dishes where anti-mouse antibody was bound. Hydroxyethyl methacrylate copolymer microspheres were conjugated with monoclonal antibodies directed against surface markers of human lymphocytes or with anti-mouse immunoglobulin antibodies and used for surface labelling of cells. The results obtained by immunofluorescence were in good correlation with labelling by hydroxyethyl methacrylate immunobeads.

0378-4347/86/$03.50

INTRODUCTION

Analytical and separation methods used for phenotypical and functional characterization of mixtures of cells from lymphoid tissues have contributed considerably to the understanding of the role of cell interactions during the immune response. Both non-specific and specific procedures have been used lately to separate subpopulations of lymphocytes and macrophages. One of the approaches most suited for specific separation of immunocompetent cells involves methods based on affinity chromatography [1–3]. Antibodies reacting with the membrane markers of appropriate cells and fixed to a solid carrier are used to bind the cells to the immunosorbent.

The development of monoclonal antibodies with an exactly defined reactivity represented another major advance in the analysis of cells participating in immune reactions [4, 5]. Visualization of membrane antigens by these monospecific reagents is most often done by immunofluorescence or immunoenzymatically. The method to be increasingly used in recent years is the labelling by polymeric particles with a bound ligand [6, 7]; the particles form so-called rosettes with appropriate cells.

Our work concentrated on comparing separation techniques based on affinity chromatography on immunosorbents of different types with bound poly- and monoclonal antibodies. The fractionated cells were characterized using a set of monoclonal antibodies against T cells. The functional capacity of the separated cells was tested by incorporation of [^{3}H] thymidine after stimulation of cells cultivated with T and B mitogens. Surface labelling was done by immunofluorescence and immunoenzymatic methods and also, for comparison, by polymeric particles with bound antibodies.

EXPERIMENTAL

Immunosorbents

Hydroxyethyl methacrylate (HEMA) Separon gel was prepared by heterogeneous suspension copolymerization of 2-hydroxyethyl methacrylate in the presence of inert solvents, particularly cyclohexanol and dodecyl alcohol [8]. By adjusting the amount of the cross-linking monomer and the ratio of the two inert components, the gel porosity and the reactive functional groups for binding may be varied over a wide range. Separon 300 and 1000, particle size 300—400 μm, activated by cyanogen bromide or by epichlorohydrine with bound antibody was used in the experiments [9]. Likewise, cyanogen-bromide-activated Sepharose 6MB (Pharmacia, Uppsala, Sweden) was used for binding the antibodies and for subsequent separation procedures. Polystyrene bacteriological dishes (Kohinoor, Dalečín, Czechoslovakia) were used for antibody adsorption and cell separation [10, 11].

Monoclonal and polyclonal antibodies

Monoclonal mouse hybridoma antibodies included BL-T2 (pan-T antibody) from Karl Marx University (Leipzig, G.D.R.), BL 1a (pan-T) and BL 14 (helper/inducer) from Immunotech (France) and BL 15 (suppressor/cytotoxic T cell) [12].

Polyclonal antisera: pig serum against human immunoglobulins IgM + IgA + IgG (SwAHu/Ig, Institute of Sera and Vaccines, Prague, Czechoslovakia), pig serum against light chains of human immunoglobulins (SwAHu/BJL and SwAHu/BJK, Institute Sera and Vaccines) and pig serum against mouse γ-globulin (SwAM, Institute of Sera and Vaccines). Binding to immunosorbents or HEMA particles was done with purified antibodies or immunoglobulin fractions of antisera or ascitic fluid; Fab 2 was prepared from pig anti-Ig serum by pepsin cleavage [13].

Isolation and fractionation of mononuclear cells from blood and tonsils

Suspension of mononuclear cells was obtained from peripheral blood of healthy donors by centrifugation on a Verographin—Ficoll mixture with density 1.077 according to Böyum [14]. Tonsils of adult patients (diagnosis: chronic tonsilitis) were cut into pieces and homogenized in Minimal Eagle Medium (MEM, Institute of Sera and Vaccines) containing 5% foetal calf serum and 40 μg/ml gentamycin (Pharmachim, Bulgaria). The medium was also used for subsequent cell washing and fractionation procedures. Mononuclear tonsilar cells were isolated by centrifugation on a Verographin—Ficoll mixture. The cell count was determined after a triple wash of mononuclear cells from both the peripheral blood and the tonsils. Viability of the cell suspension before and after fractionation was determined by trypan blue or by propidium bromide.

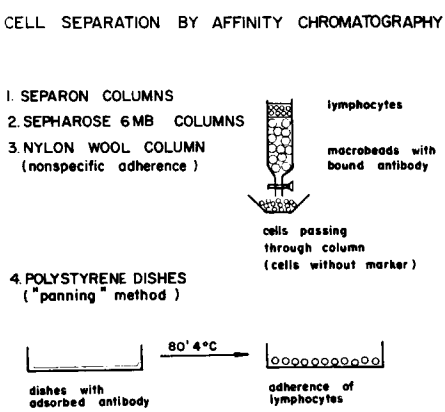

Fig. 1. Scheme of fractionation procedures.

Fractionations of mononuclear cells by affinity chromatography on Separon and Sepharose 6MB columns were performed as described previously [3, 15]. The cells were released from the immunosorbents mechanically. Fractionations on dishes with bound antibodies were carried out according to Mage et al. [10], and purifications of T cells on Nylon wool according to Greaves et al. [16]. The fractionation procedures are schematically illustrated in Fig. 1.

Relative proportion of lymphocyte subpopulations and their functional capacity before and after fractionation

The percentage proportion of lymphocyte subpopulations before and after

separation was determined by an immunofluorescence method. The numbers of B cells were assayed by direct immunofluorescence using fluorochrome-conjugated anti-Ig serum (SwAHu/Ig-FITC, Institute of Sera and Vaccines). The total numbers of T cells and that of T 4 and T 8 cells were determined by indirect immunofluorescence using the monoclonal antibodies mentioned above and a polyclonal antibody against mouse IgG labelled with fluorescein (SwAM/FITC, Institute of Sera and Vaccines).

The functional capacity of the cells was monitored via their ability to proliferate after stimulation with mitogens of T and B cells (concanavalin A, suspension of heat-inactivated bacteria *Staphylococcus aureus* Cowan I) [15]. Incorporation of [^{3}H] thymidine was measured in microcultures after a five-day cultivation [15]. The results of incorporation experiments are given as stimulation indices, i.e. the ratio of cpm of a culture stimulated with a mitogen to that of a non-stimulated culture.

Surface labelling of lymphocytes using HEMA particles and the immunoperoxidase method

Particles based on 2-hydroxyethyl methacrylate copolymer were prepared according to Rembaum et al. [6] by γ-irradiation-activated copolymerization of 100 ml of a nitrogen-purged 5% aqueous solution of a mixture consisting of 2-hydroxyethyl methacrylate (77.9%, w/w), ethylene dimethacrylate (0.1%,

TABLE I

FRACTIONATION OF TONSILAR LYMPHOCYTE SUBPOPULATIONS ON COLUMNS

Separation column	Cell fraction	Percentage of cells*				Viability (%)
		sIg+**	T_1+***	T_4+***	T_8+***	
–	Unseparated	25.1	74.5	64.5	11.2	98.5
Anti-human Ig Separon	Non-adherent	2.4	97.6	80.4	15.9	97.2
	Adherent	36.5	47.3	N.D. §	N.D.	45.4
Fab 2 anti-human Ig Separon	Non-adherent	1.8	96.4	81.3	14.4	98.3
	Adherent	45.7	54.3	N.D.	N.D.	48.2
Anti-human Ig Sepharose 6MB	Non-adherent	1.8	93.8	82.5	13.6	96.4
	Adherent	42.1	38.7	N.D.	N.D.	52.5
Nylon wool	Non-adherent	1.1	97.4	75.3	21.4	97.5
Pan-T cell Separon	Non-adherent	60.4	15.8	N.D.	N.D.	94.3
Anti-mouse Ig Separon	Non-adherent §§	78.3	9.3	N.D.	N.D.	96.7

*Arithmetic means from results of five separations.
**Direct immunofluoresence for B cell determination.
***Indirect immunofluorescence (monoclonal antibodies against T cell subsets were used).
§N.D. = Not done.
§§Cells were treated with monoclonal pan-T cell antibody before fractionation.

w/w), methylenebisacrylamide (2%, w/w) and methacrylic acid (20%, w/w) [17]. These particles were activated by carbodiimide for antibody binding according to Molday et al. [7]. The cells were labelled either directly or indirectly. In the former case, 10^6 cells were supplemented with 10^8 particles in 200 μl of MEM, centrifuged for 3 min, and then incubated for 60 min or overnight at 4°C. They were then carefully resuspended and the number of rosettes was determined in a microscope using toluidin blue. The indirect method consisted of a 30-min incubation of the cells at 4°C with a monoclonal antibody, triple wash and mixing the cells with HEMA particles with bound anti-mouse antibody as in the direct method. Cells with bound microspheres were scored in smears after May—Grünwald—Giemsa staining. Cells with three or more particles were considered positive.

The enzyme immunoassay for determination of the subpopulations of human lymphocytes was performed according to De Cock et al. [18].

RESULTS

The composition of the cell suspension obtained after separation on columns packed with Nylon wool, Separon with bound anti-Ig antibody or its Fab 2 fragment, with bound monoclonal pan-T antibody, and with Sepharose 6MB with bound anti-Ig antibody was tested by immunofluorescence with mono-

TABLE II

FRACTIONATION OF TONSILAR AND BLOOD LYMPHOCYTES ON DISHES (PANNING)

Origin of cells	Antibody adsorbed	Cell fraction	Percentage of cells*				Viability (%)
			sIg+**	T_1+***	T_4+***	T_8+***	
Tonsils	-	Unseparated	27.5	69.3	58.7	12.6	97.3
	Anti-human L chain	Non-adherent	2.4	92.6	79.5	16.8	94.4
		Adherent	34.2	N.D. §	N.D.	N.D.	30.4
	Pan-T cell	Non-adherent	41.3	43.4	N.D.	N.D.	98.3
		Adherent	N.D.	95.4	N.D.	N.D.	41.2
Blood	—	Unseparated	10.3	70.3	45.5	18.2	99.1
	Fab 2 anti-human Ig	Non-adherent	1.2	91.2	61.2	28.7	97.2
		Adherent	50.4	N.D.	N.D.	N.D.	25.4
	Pan-T cell	Non-adherent	32.5	48.2	N.D.	N.D.	95.7
	Anti-mouse Ig	Non-adherent §§	64.3	12.7	N.D.	N.D.	96.8

*Arithmetic means from results of five separations.
**Direct immunofluorescence for B cell determination.
***Indirect immunofluorescence (monoclonal antibodies against T cell subsets were used).
§N.D. = Not done.
§§Cells were treated with monoclonal pan-T cell antibody before fractionation.

clonal antibodies. Table I shows that the degree of purity of T cells separated on an anti-Ig Separon column, Sepharose 6MB column and Nylon wool column is comparable and exceeds 90% in all three cases. It should be noted that a relative accumulation of T 8 cells was found on the Nylon wool column. The degree of concentration of B cells on Separon with bound monoclonal pan-T antibody was lower than with cells pretreated with monoclonal antibodies before fractionation on Separon with bound anti-mouse antibody (Table I).

Separation on polystyrene dishes with adsorbed antibody (so-called panning) appears to be highly efficient. As with the columns, the separation of cells pretreated with monoclonal antibodies was better than on dishes with bound antibody (Table II). Separations on columns as well as panning on dishes preserved a high degree of viability of non-adhering (passing, washed-out) cells, whereas the viability of cells adhering to the gel or the dish and obtained mechanically was very low (Tables I and II).

Tests of functional capacity of cells after separations showed retention of proliferative activity in non-adhering cells (Figs. 2 and 3). The degree of incorporation of [^{3}H]thymidine depended on the mitogen used (B,T mitogen) and corresponded well to the depletion or accumulation of T and B cells (Figs. 2 and 3) observed by immunofluorescence.

Surface labelling by the HEMA particles with bound antibody was comparable with cell labelling by immunofluorescence or peroxidase. This was

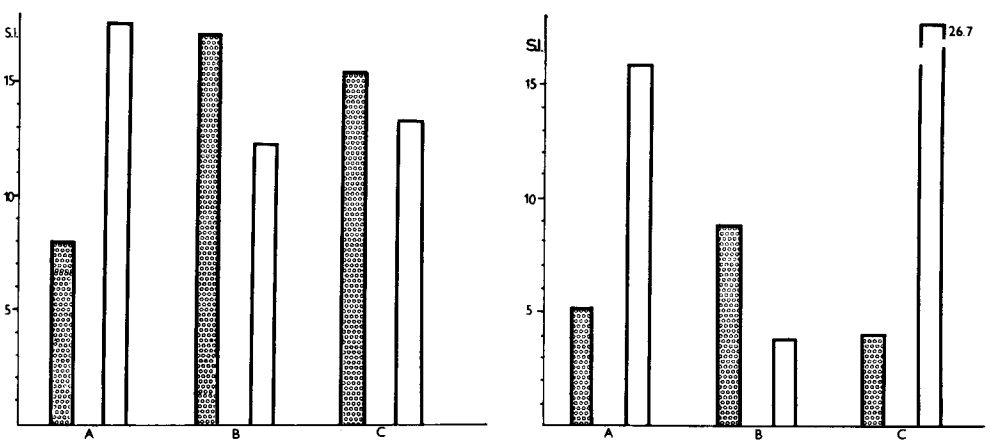

Fig. 2. [^{3}H]Thymidine incorporation by human tonsilar cells after fractionation on anti-human Ig Separon and Nylon wool columns and in vitro mitogenic stimulation. (▣) Response to concanavalin A; (□) response to bacterial suspension of *Staphylococcus aureus* Cowan I. (A) Unseparated cells ; (B) anti-human Ig Separon column passed cells; (C) Nylon wool column passed cells. S.I. = Stimulation index (arithmetic means from three to six cultures).

Fig. 3. [^{3}H]Thymidine incorporation by human peripheral blood cells after panning on polystyrene dishes and in vitro mitogenic stimulation. (▣) Response to concanavalin A; (□) bacterial suspension of *Staphylococcus aureus* Cowan I. (A) Unseparated cells; (B) cells non-adherent to anti-human Ig dishes; (C) cells pretreated with pan-T cell monoclonal antibody and fractionated on anti-mouse Ig dishes (non-adherent fraction). S.I. = stimulation index (arithmetic means from three cultures).

TABLE III

SURFACE LABELLING OF HUMAN BLOOD LYMPHOCYTES USING HEMA IMMUNOBEADS, IMMUNOFLUORESCENCE AND IMMUNOENZYMATIC ASSAY

Results represent arithmetic means of six estimations.

Preincubation medium	Particle coating*	Percentage of cells		
		HEMA-labelling	FITC-labelling	Peroxidase-labelling
—	Anti-human Ig	11.2	9.4	10.2
—	Pan-T cell Ab	54.3	N.D.**	N.D.
BL 1a (Pan-T)	Anti-mouse Ig	68.5	64.5	70.1
BL 4 (T 4)	Anti-mouse Ig	44.2	47.3	45.2
BL 15 (T 8)	Anti-mouse Ig	26.4	28.7	30.5
BL 1a (pan-T)	Bovine albumin	0.1	N.D.	N.D.
—	Anti-mouse Ig	0.2	0.1	1.2

*Alternatively, fluorescein isothiocyanate (FITC)- or peroxidase-labelled antisera were used.
**N.D. = Not done.

especially true in experiments with indirect labelling in which cells pretreated with monoclonal antibody and washed, reacted with HEMA particles with bound anti-mouse antibody (Table III).

DISCUSSION

Identification and separation of subpopulations of human lymphocytes differing in their antigenic determinants and functional capacity is crucial for diagnostics, prognosis and therapy of some lymphoproliferative disorders, immunodeficiencies and other immunologically mediated diseases. The advantages of monoclonal antibodies prepared against differentiation markers of human lymphocytes [19] for characterization of human lymphocytes are widely known. Monoclonal antibodies are used for human cell separation on the fluorescence-activated cell sorter or for separations using complement-mediated cytolysis. Our study pointed out the usefulness of these reagents also in other separation systems. Our results indicate the advantages of using columns or dishes with bound anti-mouse antibody for separation of cells pretreated with monoclonal antibodies. From the methods recently used for separation of lymphoid cells [20, 21], the method of panning appears the most convenient; it is fast, requires no sophisticated equipment and ensures sterile working conditions. However, under our conditions, cells adhering to the immunosorbent were released only with difficulty and most were damaged [21]. Certain possibilities are offered by the method of cell release by excess anti-mouse antibody used in a similar system [22]. It seems that hydrophobic rather than ionic interactions may play a key role in non-specific cell binding to solid supports, a fact which conveniently can be used for probing and separation of cells [23, 24].

The effect of enrichment of T or B cells was clearly visible also in the response to concavalin A (T mitogen) and to a heat-inactivated suspension of *Staphylococcus aureus* Cowan I bacteria containing the SpA (B mitogen).

Polymeric particles have lately been used as immunochemical reagents, notably polystyrene latex particles which, however, have the shortcoming of non-specifically sticking to cell surfaces. Rembaum et al. [6] synthesized microspheres by polymerization of methacrylate monomers. The resulting particles have a minimal non-specific stickiness and can be readily used for binding antibodies by various chemical procedures [6, 7]. Our study proved the suitability of analogously prepared particles for detection of surface antigen of human lymphocytes, in particular using monoclonal antibodies and particles with bound anti-mouse antibody. The method of labelling by particles facilitates also the morphological evaluation of labelled cells, provided that staining of the smear is carried out.

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