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Heteroantisera prepared against B lymphocytes at different stages of differentiation

I. PREPARATION OF SERA AND CYTOTOXICITY TO LYMPHOID CELLS FROM DIFFERENT ORGANS*

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Summary. A routine method is described whereby anti-mouse B-cell antisera can be raised in rabbits. As judged by trypan blue dye exclusion these sera have negligible cytotoxicity to murine T lymphocytes but seem to detect differences in non-T lymphocytes in different organs. By sedimentation analysis some of the sera may detect (in addition to mature B cells) earlier precursors of mature B lymphocytes in bone marrow and spleen.

INTRODUCTION

Mature lymphocytes can be divided into two distinct subclasses, B and T cells, each of which has documented surface markers which allow their unequivocal identification (Greaves, Owen & Raff, 1973). In addition, lymphocyte differentiation occurs in three biologically distinct phases, from stem cells (pluripotent) to mature antigen-committed lymphocytes (with the ability to respond to foreign antigen and proceed to further differentiation) and finally to effector cells (antibody-producing cells, cytotoxic lymphocytes etc). During recent years considerable effort has been spent in attempting to characterize the various stages in differentiation along the lymphocyte pathway. Progress has been made in assigning distinct physical parameters to T or B cells, and to both the precursors of these and their resultant effector cells (Shortman, 1974). Functional assays (which depend upon the biological activity which these cells are programmed to express) have also been developed which characterize the cells (Gorczynski, Miller & Phillips, 1971; Lafleur, Miller & Phillips, 1972). However, little data is available on any unique surface antigenic properties associated with the different stages of differentiation of lymphocytes.

For B-lymphocyte differentiation, it is known that the PC-1 antigen is associated only with plasma cells (Takahashi *et al.*, 1971). The majority of studies of B-cell differentiation have merely investigated the different degrees of expression of those antigens known to exist on the mature committed B cell (e.g. immunoglobulin, Fc and complement receptors). Using these markers Osmond & Nossal (1974) have indicated evidence for an increase in surface immunoglobulin from stem cells to mature B lymphocytes. Subpopulations of such mature cells also exist with/without complement receptors, though both seem to be functionally active with

* The author regrets that no reprints of this manuscript will be available.

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perhaps complement receptors stabilizing T–B interaction (Parish & Chilcott, 1975).

Until recently T-lymphocyte differentiation was described in terms of density of thy-1 antigen (Shortman & Jackson, 1974) and expression of TL (Schlesinger, 1972) though now it appears that anti-Ly sera will enable selection at least of different subclasses of mature T lymphocytes, and perhaps their resultant effector cells (Cantor & Boyse, 1975).

We have begun an investigation into the ability of heteroantisera (raised to murine tissue in rabbits) to distinguish B lymphocytes at different stages of differentiation, and perhaps also to distinguish different subclasses of mature committed B cells. The data presented below describe the preparation of these sera and compare the cytotoxicity of the sera for cells with discrete organ distribution and physical size. In the subsequent paper we have investigated the effect these sera have on the biological activities expressed by the cells of the B-lymphocyte axis of differentiation.

MATERIALS AND METHODS

Mice

BALB/c, C3H/ANF, DBA/2 and CBA mice from Cumberland View Farms, Clinton, Tennessee, U.S.A. were used interchangeably in these experiments. Male and female mice were used.

Cells

Spleen, thymus and bone marrow cells were prepared as described earlier (Gorczynski *et al.*, 1971). Peripheral lymph node cells (axillary and inguinal nodes only) were prepared in similar fashion.

Cell separation

(i) Velocity cell sedimentation (Miller & Phillips, 1969) was performed in a sterile glass sedimentation chamber (model SP-120, John's Glass, Toronto, Canada) at 4°. The cells were loaded in 0·3 per cent bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and a buffered step gradient from 0·6 per cent BSA in PBS to 2 per cent BSA in PBS was used. Cells were sedimented for 5 h at 4° and fractions corresponding to 0·7 mm/h were collected. Cells were centrifuged (200 *g* for 5 min at 4°) and resuspended in α medium supplemented with 5×10^{-5} M 2-mercaptoethanol and 10 per cent foetal calf serum (α F_{10}).

(ii) Nylon wool columns were used to prepare lymphoid cell suspensions enriched for T cells and depleted of B cells. The technique used was essentially that described by Julius, Simpson & Herzenberg (1973). Nylon-Wool adherent cells (enriched for B cells) were obtained by mechanical agitation as described by Trizio & Cudkowicz (1975).

(iii) AKR anti-C3H thymus cell antiserum (anti-Thy 1·2) was used to deplete mouse lymphoid suspensions of mature T lymphocytes. Cells were incubated in α F_{10} with anti-Thy 1·2 (final concentration 1/15) at a concentration of 10^7 cells/ml for 1 h at 4°. The cells were washed and re-incubated in α F_{10} for a further 45 min at 37° in rabbit complement (mouse spleen cell absorbed). The final complement concentration was 1/10. The cells were then washed twice and resuspended in α F_{10}. Functional characteristics of this serum are described elsewhere (Gorczynski, 1976).

Preparation of heteroantisera

(i) Anti-mouse immunoglobulin (AMI_g). Crude mouse immunoglobin was precipitated from normal mouse serum with 50 per cent saturated $(NH_4)_2SO_4$. The preparation was resuspended in PBS and 10 mg in Freund's complete adjuvant injected s.c. into two rabbits. The injection was repeated twice, at 2-weekly intervals, and the rabbits bled out 10 days after the last injection. The sera were pooled, heat inactivated, and stored at −20°. All AMI_g was absorbed five times with an equal volume of mouse thymocytes prior to use.

(ii) Anti-lymphocyte serum (ALS). Rabbits were injected i.v. twice, 14 days apart, with 2×10^8 mouse thymocytes. The rabbits were bled by cardiac puncture under ether anaesthesia and the serum so collected was heat-inactivated. $F(ab')_2$ fragments of the IgG portion of this serum (from Sephadex G-200) were prepared as follows. Four hundred milligrams IgG were digested with pepsin (2500 u/mg, Sigma Chemical Company St. Louis, Missouri) at an enzyme-substrate ratio of 1:50 in acetate (0·08 M) saline (0·10 M) buffer, pH 4·5, for 20 h at 37°. The reaction was stopped by raising the pH to 8·0 and the material was dialysed against three changes of 200 vol. PBS for 24 h at 4°. The preparation was then applied to a $50 \times 2{\cdot}0$ cm Sephadex G-200 column. Three peaks were obtained: undigested IgG (28 per cent), $F(ab')_2$ (40 per cent) and a smaller peak containing Fc fragments (32 per cent). The $F(ab')_2$ fragments were adjusted to a concentration of 10 mg/ml.

All subsequent heteroantisera were prepared as follows. Spleen, peripheral lymph node and bone marrow cells were removed from twenty non-immune donor mice (8–10 weeks of age). The cells were treated with anti-Thy 1·2 and rabbit complement as described above, and, after washing twice with cold PBS, the viable cell count (trypan blue dye exclusion) for each suspension was determined. 1×10^8 of each of the cells were then incubated in 2 ml ALS-F(ab′)$_2$ (see above) for 90 min at 4°. Following this the cells were washed twice, resuspended in 2 ml cold PBS and injected i.v. into adult recipient rabbits (20 weeks of age). The injections were repeated 2 weeks later and the rabbits bled 10 days after the last injection. The sera were heat-inactivated and stored at −20° until use. The rabbit recipients had themselves been injected within 2 h of birth with 1×10^9 mouse thymocytes and within the following 4 days received three subsequent injections of 1×10^9 cells each. (Using this latter protocol we found the rabbits made a lower titre of ALS antibody (requiring two to three less absorptions with packed cells for complete removal) than if no such injections were given). The purpose of all of these manipulations (i.e. anti-thy 1·2 treatment, coating with F(ab′)$_2$ of ALS and injection into rabbits made neonatally tolerant of mouse thymocytes) was to decrease recognition of 'mouse lymphocyte antigens', and increase the likelihood of detecting antibodies to more restricted B-cell determinants (organ distribution specific; differentiation state specific etc.).

(iii) Anti-spleen B-cell antibody (ASB). Rabbits were injected (as above) with ALS-F(ab′)$_2$ coated spleen cells. The serum was absorbed five times with an equal volume of packed mouse thymocytes prior to use.

(iv) Anti-peripheral lymph node B-cell antibody (APB). Peripheral lymph node cells were used as the immunizing cells. Again these sera were absorbed five times with an equal volume of packed mouse thymocytes prior to use.

(v), (vi) and (vii) Anti-whole bone marrow (ABM), antibone marrow B cell (ABMB) and antibone marrow stem cell (ABMSC) were prepared as follows. 4×10^8 anti-Thy 1·2-treated bone marrow cells were fractionated for 4 h at 4° by velocity sedimentation. Cells sedimenting in the region 2·5–3·5 mm/h (bone marrow B lymphocytes) (Miller & Phillips, 1970) were collected and pooled, as were cells sedimenting in the region 5·0 to 7·0 mm/h (stem cells and others (Miller, Gorczynski, Lafleur, Macdonald & Phillips, 1975)). 1×10^8 cells of each of these pools, along with 1×10^8 unfractionated (whole bone marrow) cells were incubated in ALS-F(ab′)$_2$ and injected into rabbits as above. The sera collected from the rabbits in these three cases were absorbed five times with packed thymus cells.

Trypan blue dye exclusion tests (two-step)

All cells to be tested with the relevant antisera were treated at a final concentration of 10^7 cells/ml in α F_{10}. Cells were incubated in 100 μl containing the required antibody at the relevant concentration for 60 min at 4°. The cells were centrifuged (200 *g* for 5 min at 4°) in 1 ml PBS, resuspended in 100 μl spleen-cell absorbed rabbit complement (final dilution 1/10 α F_{10}) and incubated for a further 45 min at 37° in a humidified atmosphere with 10 per cent CO_2. The cells were then chilled on ice, 100 μl of trypan blue dye added, and after a period of 10 min the number of dye-excluding cells determined. This value was compared with the number after incubation in medium throughout to determine specific cytotoxicity. Controls were included in which cells were incubated in antibody but no complement or medium (no antibody) and then complement only. All data are expressed as a percent cytotoxicity thus:

$$100 \times \left[1 \frac{\text{(number viable cells in test suspension)}}{\text{(number viable cells in medium only)}}\right].$$

RESULTS

Cytotoxicity of antisera to unfractionated cells from thymus, spleen peripheral lymph node or bone marrow

Since our primary intention was to raise antisera to antigens unique to the B-cell series (either differentiation antigens on cells of different maturity, or organ specific antigens on cells at similar stages in differentiation) we were naturally interested in investigating the cytotoxicity of the antisera to T cell-depleted cell populations. Although the sera had been previously absorbed to remove T-cell activity, only by testing T-depleted cells could we be sure that the cytotoxicity seen was not due to T-cell killing. Accordingly, we prepared spleen, peripheral lymph node and bone marrow from five normal C3H mice

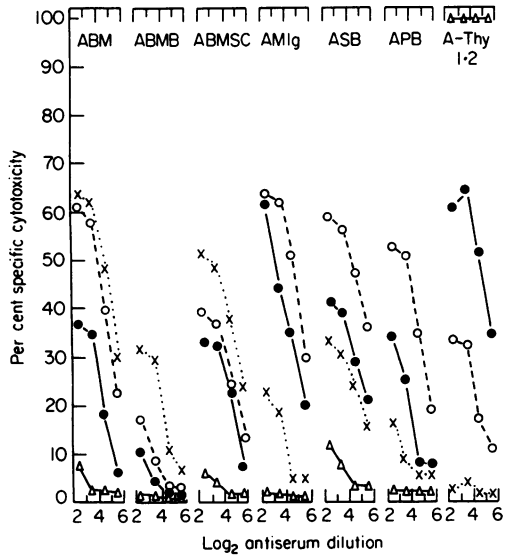

Figure 1. Cytotoxicity of heteroanti-B sera to anti-Thy 1·2 treated spleen (○), lymph node (●) or bone marrow (×) cells, or to untreated thymocytes (△). All cytotoxicity tests were performed as described in the Materials and Methods section, where a description of the antiserum nomenclature is found.

and treated the cells with anti-Thy 1·2 serum (1/15 final concentration) and complement as described earlier. An aliquot of the cells was left untreated, as was a preparation of thymus cells from the same mice. Each of these treated populations and the untreated thymus cells were tested with varying concentrations of the heteroantisera described in the Materials and Methods section. The aliquots of untreated cells and thymus cells were tested with various concentrations of anti-Thy 1·2 with complement. The data showing the cytotoxicity of these antisera to the different cell populations are shown in the panels of Fig. 1 (representative data from one of four experiments).

All of the absorbed sera had little or no cytotoxicity to murine thymocytes in Fig. 1. In contrast, and as expected, the anti-Thy 1·2 serum killed 100 per cent of thymus cells at all dilutions tested. Similarly, the anti-Thy 1·2 serum killed 60–65 per cent of peripheral lymph node cells and 35–36 per cent of spleen cells at a 1/5–1/15 dilution with little cytotoxicity to bone marrow cells at this concentration. These figures corresponded well with earlier published data pertaining to the relative mature B/T cell frequency in these cell populations (Raff, 1969).

Tests performed on T cell-depleted lymphoid populations with the heteroantisera were also instructive. The cytotoxicity seen with the various sera varied markedly according to the lymphoid population under test. Thus AMIg was most cytotoxic towards spleen and peripheral lymph node, and rather less so towards bone marrow. This was in agreement with fluorescence data using fluorescein-conjugated rabbit-anti-mouse Ig (unpublished), though the peak cytotoxicity in all cases (e.g. 60 per cent for spleen) was rather less than expected from the fluorescence index (75–80% for anti-Thy 1·2-treated spleen cells). Similarly, the ASB and APB sera were most cytotoxic towards spleen cells and less so to lymph node and bone marrow. In particular, APB was considerably less cytotoxic to bone marrow than ASB. All sera raised against either unfractionated bone marrow (ABM) or fractionated bone marrow (ABMB, ABMSC) were most cytotoxic towards bone marrow. Only ABM and ABMSC showed highly significant cytotoxicity towards spleen and lymph node cells. Perhaps most surprising was the activity of ABMSC with lymph node cells, since the latter population (unlike spleen and bone marrow) is reported to have very few cells with sedimentation velocity greater than 5 mm/h (the pool which includes stem cells for lymphpoiesis and hemopoiesis, Miller *et al.*, 1975).

Cytotoxicity of antisera to fractionated spleen, lymph node, and bone marrow cells

The data above indicated that our heteroantisera could detect organ-specific differences in non-T lymphocytes prepared from bone marrow, spleen and peripheral lymph node. Further analysis of the cellular specificity of the sera was obtained using populations from lymph node, spleen and bone marrow which had been previously fractionated by nylon wool adherence (to enrich for T lymphocytes (Julius *et al.*, 1973)), or velocity sedimentation a procedure known to separate cells with different biological activities (Shortman, 1974).

Spleen and lymph node cells were prepared from 4 non-immune BALB/c mice and cell suspensions made as before. 1×10^8 cells of each population were applied to nylon wool columns and incubated at 37° for 45 min. The cells were eluted with 25 cm^3 of warm (37°) PBS with 5 per cent foetal calf serum. Column adherent cells were obtained by mechanical

Table 1. Effect of nylon-wool column separation on cytotoxicity of heteroantisera to mouse spleen and peripheral lymph node cells

Antiserum* used in test	Percent cytotoxicity†					
	Spleen cells			Peripheral lymph node cells		
		Nylon-wool column‡			Nylon-wool column‡	
	Unseparated	Effluent	Adherent	Unseparated	Effluent	Adherent
ABM	34±3	11±2	47±6	22±4	6±2	25±8
ABMB	12±4	6±2	n.d.	14±2	4±3	n.d.
ABMSC	20±3	2±2	n.d.	24±4	6±2	n.d.
AMIg	49±7	19±3	67±6	32±3	7±1	n.d.
ASB	38±9	21±3	65±13	31±5	10±2	49±10
APB	24±4	10±3	47±4	19±2	8±4	32±4
Anti-Thy 1·2	32±6	74±9	5±2	63±6	89±5	10±3

n.d. = Not determined.

* All antisera were used at a final concentration of 1/10. The technique for treatment with serum and rabbit complement was described in the Materials and Methods section.

† Percent cytotoxicity (arithmetic mean ± s.e. derived from three independent counts) to cells taken from four non-immune BALB/c mice. Non-specific cytotoxicity due to rabbit complement alone or serum alone was less than 5 per cent in all cases.

‡ Nylon-wool column separation was described in the Materials and Methods section. Recovery of spleen and lymph node cells in the column effluent was 22 and 63 per cent respectively, and in the column absorbed preparation 61 and 25 per cent respectively.

disruption. Both cell populations were then spun down at 200 *g* for 5 min at 4°. These T-cell enriched, T cell-depleted and unfractionated cells were then treated as before with the antisera described in Fig. 1. Only one concentration (a 1 in 10 dilution) was used in this test, but all groups were set up in triplicate. The data from one such experiment (of three) showing the cytotoxicity of the various antisera are shown in Table 1.

As judged by the effect of anti-Thy 1·2 serum and the cell recovery from the column, the column effluents from both the spleen and lymph node preparation were substantially enriched for T lymphocytes, while the column adherent cells were depleted of such cells. Similarly, in all cases, the heteroantisera had considerably less affect on the nylon wool effluent cells of both spleen and lymph node than on the unseparated cell populations. Where tested, the converse was true for the column adherent cells. According to these data then the activity of all of the heteroantisera was not principally directed against T lymphocytes.

Since there was abundant evidence in the literature that cells with different biological activities have different physical properties (e.g. size, density, surface charge etc.) we were interested in investigating the effects of the antiserum described on cells of different-size. Spleen, lymph node and bone marrow cells were obtained from thirty non-immune C3H/ANf mice and treated with anti-Thy 1·2 serum and complement as before. 2×10^8 viable cells of each type were then sedimented for 4 h at 4° and fractions differing in sedimentation velocity by 0·7 mm/h were collected. Cells from populations with different sedimentation velocity were then treated with the heteroantisera described to determine the cytotoxicity of the antisera to the different cell populations. Typical data (one of two experiments) are shown in Fig. 2. In addition this figure shows data from a separate experiment in which the cytotoxicity of anti-Thy 1·2 serum to non-treated (before sedimentation) populations of spleen, lymph node and bone marrow was investigated.

The sedimentation profile of anti-Thy 1·2 sensitive cells correlated well with other data investigating the sedimentation profile of functional T cells (Miller *et al.*, 1975). Studies investigating the sedimentation profile of functional B cells (precursors of antibody-producing cells) suggest a peak of 3·0 mm/h for T-dependent B cells and a slightly

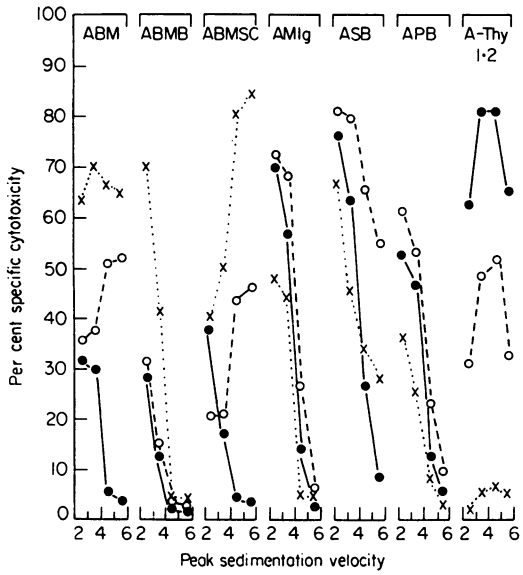

Figure 2. Cytotoxicity of heteroanti-B sera to anti-Thy 1·2 treated, velocity sedimented, spleen, bone marrow or lymph node cells. The final column shows the data obtained using anti-Thy 1·2 sera with untreated, velocity sedimented cells. The symbols are described in detail in the legend to Fig. 1.

broader profile for T-independent B cells (Gorczynski & Feldman, 1975). Early progenitors of B and T cells sediment in the region of 4–5 mm/h while uncommitted stem cells, granulocytes, macrophages etc. sediment in the large cell region (greater than 4·5 mm/h) (Lafleur *et al.*, 1972; Miller *et al.*, 1975).

As judged by cytotoxicity to velocity sedimented cell populations ABMB, AMIg and probably APB were cytotoxic primarily to B cells, though there were subtle organ differences. Thus AMIg was more cytotoxic towards spleen and lymph node than bone marrow (in keeping with the fluorescence of these cell populations with fluorescence-labelled anti-Ig (Osmond & Nossal, 1974)). Similarly APB cross-reacted more with spleen than with bone marrow while ABMB seemed equally active with small cells from both the spleen and lymph node though by far the greatest reactivity was to small marrow cells.

The ABM and ABMSC sera seemed to detect quite similar cell populations, though the reacting cells in spleen, bone marrow and lymph node were not the same. Thus predominantly larger splenic cells were killed (non-B cells?) while mainly small lymph node cells were killed (B cells). Bone marrow cells of all sizes reacted, though ABMSC seemed to detect primarily large cells in bone marrow and ABM showed similar cytotoxicity to large and small bone marrow cells. ASB serum was highly cytotoxic towards smaller cells in all organs tested, though appreciable activity to large spleen and bone marrow cells was also detected with this serum.

DISCUSSION

Considerable data has come to light in recent years on the heterogeneity involved in B-cell differentiation. Thus, by assays of immunocompetence, committed progenitors of B cells and mature B lymphocytes coexist in spleen and bone marrow, with these cells being separable in terms of physical size (Lafleur *et al.*, 1972). Strober (1975) has described differences in migration patterns, turnover rates, and tissue distribution for normal B cells and memory B cells in the rat. By examining size and surface immunoglobulin, further subpopulations of virgin B cells in different organs were detected (Strober, 1975). Gorczynski & Feldmann (1975) have indicated a difference in the size of mature B cells responding to T-dependent and T-independent antigens and Gronowicz & Coutinho (1976) have suggested that B-cell differentiation may be correlated with mitogen responsiveness. In view of the evidence that T-cell differentiation is associated with distinct membrane antigen changes (Shortman & Jackson 1974; Cantor & Boyse 1975), and that B-cell differentiation seems certainly associated with changes in density of surface immunoglobulin (Osmond & Nossal 1974) we examined whether B-cell differentiation was also accompanied by other surface changes which could be detected with the appropriate hetero-antisera

Our data (see Table 1) indicated that the reactivity of the sera described herein was indeed primarily directed against non-T lymphocytes (nylon-wool adherent cells). Moreover, studies on unfractionated cells from different lymphoid organs (Fig. 1) showed that the sera were not directed against the same cells since the peak cytotoxicity with different sera and the same lymphoid cell populations varied, and the cytotoxicity of a given serum varied according to the lymphocytes under test. These data were investigated further after fractionation of the cells under test by velocity sedimentation (Fig. 2).

After this analysis we concluded that small (B?) lymphocytes of spleen and lymph node showed

marked similarity with all sera tested save ABMSC which surprisingly indicated a cross reaction between marrow and lymph node cells. In contrast small bone marrow (B?) lymphocytes possessed antigens not found (to the same degree) on spleen and lymph node cells (see data with ABMB). Moreover, there was evidence that sera raised against spleen B cells detected small lymphocytes of all organs equally well, whereas sera raised against lymph node B lymphocytes did not detect marrow B cells as well as ASB. Finally, ABM, ABMSC and to a lesser degree ASB detected large cells in marrow and spleen which were absent in lymph node.

Further comparison of these sera, investigating their affects on functional properties of B cells (stimulation to antibody production, mitogen stimulation etc.) are described in the following paper. This also contains a comparison of our sera with other reputed heteroanti-B sera.

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