SEPARATION OF ANTIGEN-SPECIFIC LYMPHOCYTES

I. Enrichment of Antigen-Binding Cells* ‡

By W. HAAS§ and JUDITH E. LAYTON

(From the Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Victoria, Australia)

Lymphocytes are heterogeneous in structure and function. A direct approach to analyze the complexity of the immune system is to separate its key components and to study their properties. Various techniques have been developed for fractionation of lymphocyte subpopulations based on differences in density, size, adherence properties, or electrophoretic mobility (for review see 1). However, these methods are incapable of separating lymphocytes on the basis of specificity. In the past 10 years several methods have been developed for isolation and enrichment of antigen-specific cells: (*a*) Purification of rosette-forming cells (RFC)[1] by velocity sedimentation (2, 3) or density gradient centrifugation (4, 5). (*b*) Isolation of antigen-binding cells by an electronic cell sorter (6, 7). (*c*) Adsorption of specific cells to various specifically derivatized matrices and recovery (8–16).

RFC from normal (2, 5) and immunized mice (3, 4) have been separated with various degrees of success. High purification of SRBC-RFC has been achieved (4); however, it demanded a complicated and time consuming separation procedure. A disadvantage common to all methods of separation of specific RFC is that they include cells specific for more than one antigenic determinant. High enrichment of antigen-binding cells from immunized mice has been obtained also by separation of fluorescein-labeled cells in an electronic cell sorter (6, 7). However, this machine can purify only cells specific for fluorescent haptens and it is restricted by the small number of cells which can be separated in a reasonable time.

Separation of specific lymphocytes using immunoadsorbents was introduced by Wigzell (8). Nonspecific adsorption of lymphocytes to glass or plastic surfaces was a major problem in earlier attempts to enrich rather than deplete antigen-specific cells (8, 9). This problem has been markedly reduced by the use of more inert matrices such as polyacrylamide (11) or polysaccharide beads (12–14). The contact time between cells and

* This work was supported by the following grants to Professor G. J. V. Nossal: National Health and Medical Research Council and the Australian Research Grants Committee, Canberra, Australia; by NIH grant AI-O-3958, U. S. Public Health Service and by contract NO1-CB-23889 with the National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare; by the Deutsche Forschungs Gemeinschaft (grant no. Ha 723/4), Germany, and by the Volkswagen Foundation grant no. 112147.

‡ This is publication no. 2050 from the Walter and Eliza Hall Institute of Medical Research.

§ Holder of a postdoctoral fellowship (no. Ha 723/5) of the Deutsche Forschungs Gemeinschaft, Germany.

[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; BSA, bovine serum albumin; FCS, fetal calf serum; HEM, Hepes-buffered Eagle's medium; NIP, 4-hydroxy-3-iodo-5-nitrophenylated; PBS, phosphate-buffered saline; POL, polymerized flagellin; RFC, rosette-forming cells.

adsorbent is also important in reducing nonspecific binding. Nonspecific binding of lymphocytes was negligible when cells were filtered through columns of large polyacrylamide beads with a rapid flow rate (11) or when cells were incubated with antigen-derivatized nylon fibers on a relatively fast moving reciprocal shaker (15).

The second major problem of specific cell separation using immunoadsorbents is the recovery of specifically adsorbed cells. Elution of specific cells by free hapten or antigen would be the ideal method of recovery. However, the general experience has been, with exceptions (11, 16), that cells once bound to an immunoadsorbent could not be eluted by free antigen (9, 15, 17). Recently we described a new method using gelatin-coated tubes as an immunoadsorbent for separation of specific lymphocytes (18). Hapten groups can be coupled covalently to gelatin without affecting its ability to form an insoluble gel at low temperature. The advantage of this method is that bound cells can be recovered by melting the gel at physiological temperatures. In the present article we describe a modification of this method which has proved more effective in preparing a population of hapten-specific lymphocytes. In an accompanying paper the functional properties of the recovered cells are described.

Materials and Methods

Mice. Specific pathogen-free CBA mice (8–10-wk old) were used in all experiments.

Proteins. Bovine serum albumin (BSA) was obtained from Armour (fraction V). Bovine gelatin was obtained from Parke Davis and Company, Sydney.

Haptens and Hapten-protein Conjugates. DNP_{58}-BSA were prepared according to the method of Eisen (19). Similarly DNP-gelatin was prepared by mixing 20 ml 20% wt/vol bovine gelatin, 10 ml 8% wt/vol K_2CO_3 and 10 ml of 10 mg/ml DNP-sulphonic acid and stirring the mixture gently for 16 h at 37°C. It was then dialyzed extensively against distilled water at 4°C. The substitution ratio of this standard preparation was approximately four DNP groups per molecule of gelatin. Different substitution ratios were obtained by varying the concentration of DNP-sulphonic acid or the reaction time. The substitution ratio was calculated from the absorbency at 360 nm and 215 nm assuming an average molecular weight of 10^5 for gelatin.

NIP-gelatin was prepared according to the method of Brownstone et al. (20). 50 ml gelatin (10%) was mixed with 140 ml 0.2 M bicarbonate and 100 mg 4-hydroxy-3-iodo-5-nitrophenylated (NIP)-azide in 2 ml dimethylformamide and stirred gently at room temperature for 12 h. The mixture was then dialyzed extensively against phosphate-buffered saline (PBS) (pH 7.3). The substitution ratio was calculated from the absorbency at 430 nm and 215 nm. DNP-lysine was purchased from British Drug Houses Ltd.

Antisera

ANTIMOUSE IG. A sheep was immunized with purified MPC 86 myeloma protein (IgG 2b). The antiserum was passed over an immunoadsorbant of M 86-Sepharose and the anti-Ig antibodies were eluted with glycine-HCl buffer (pH 2.8), and concentrated by vacuum dialysis. This anti-Ig was specific for immunoglobulin by immunoelectrophoresis and gel diffusion.

ANTI-DNP IG. Serum from MOPC 315 tumor-bearing mice was heat-inactivated at 56°C for 30 min and passed over an immunoadsorbent of DNP-poly-L-lysine-Sepharose. The MOPC 315 anti-DNP IgA was rapidly eluted with 4 M urea and immediately dialyzed against borate-saline buffer pH 8.5. It was then concentrated by vacuum dialysis and gel filtered on Sephadex G-200. A small peak of high molecular weight material eluting in the void volume was discarded and the low molecular weight MOPC 315 was concentrated and used.

COATING OF DISHES WITH GELATIN. Sterile plastic petri dishes (Sterimed, 85 mm diameter) were coated on the base with about 1 ml of gelatin (5%, 40°C), hapten-conjugated gelatin (5%) or a mixture of equal volumes of unconjugated gelatin (5%) and DNP-gelatin (5%). As much as possible of the gelatin was aspirated from the edge of the dish with a Pasteur pipette so that only a very thin uniform layer remained. The coated dishes were left at 4°C overnight for the gel to set, then about 10 ml of cold sterile PBS (pH 7.3) was added to each dish. The dishes were used after 2 to 5 days storage at 4°C and several rinses with cold PBS.

Spleen Cells Suspensions. Single cell suspensions were prepared from CBA mouse spleens. Red cells were removed by incubating the cells suspension in 0.17 M NH_4Cl at 37°C for 8–10 min and damaged cells were removed as described elsewhere (21). The resulting cell suspension was 95–98% viable as determined by eosin dye exclusion.

Cell Fractionation. 3 ml of spleen cells suspended in Hepes-buffered Eagle's medium (HEM) were distributed over the surface of each gelatin-coated dish immediately after the PBS was poured off the dish. The dishes were then placed on a shaker-rocker which moved gently in two directions usually for 60 mins at 4°C. The shaker moved horizontally through 1.5 cm at 80 cycles per minute (cpm) and was mounted on a rocker which moved vertically through 20° at 20 cpm. The unbound cells were poured off and the dishes rinsed with Eisen's balanced salt solution (EBSS) several times until no unbound cells could be seen under an inverted microscope. Bound cells and unbound cells could be easily distinguished from each other since the unbound cells moved relative to the dish when the medium was agitated. For each rinse 5–10 ml of EBSS was added carefully at the side of the dish, the dish swirled a few times, then the buffer poured off and immediately replaced. Bound cells could be counted under the inverted microscope in situ or, after melting the gel with 10 ml HEM at 37°C, in a Coulter counter (Coulter Electronics Inc., Hialeah, Flor.). Several DNP-gelatin-coated dishes which contained no cells were harvested similarly and gave an average count of 500 per 0.5 ml due to dust from the dishes which could not be eliminated. This background was subtracted from all counts.

Treatment of Spleen Cells with Collagenase. Collagenase (A grade, activity 83 U/mg, Calbiochem, San Diego, Calif.) was dissolved in PBS at a concentration of 1 mg/ml, Millipore filtered and stored at 4°C.

Bound cells were recovered from gel-coated dishes in 10 ml HEM (37°C), centrifuged, pooled, washed once, and resuspended in 0.5 ml HEM containing 5% fetal calf serum (FCS). 10 μl collagenase was added and the cells were kept at 4°C for 30 min. or at 37°C for 10 min., then washed once with HEM (5% FCS) through underlayers of FCS.

Determination of Lymphocytes Bearing Easily Detectable Surface Ig. 10^6 spleen cells were incubated at 37°C for 20 min in 1 ml HEM containing 300 μg antimouse Ig which had been labeled with fluorescein (22). The cells were then washed twice with medium, resuspended in a drop of medium and put onto a glass slide for observation. Stained cells showed bright caps of fluorescent material. 500 cells were scored per sample.

Rosette-forming Cell Assay. DNP-SRBC were prepared as described elsewhere (23). SRBC with higher density of DNP were obtained by using the fivefold concentration of DNP-Fab. Similarly, SRBC were coupled with NIP-conjugated Fab prepared according to the method of Brownstone et al. (20).

0.2 ml of a 2% suspension of SRBC, DNP-SRBC, or NIP-SRBC was mixed with 0.2 ml of unfractionated spleen cells (2×10^6) or collagenase-treated hapten-gelatin binding cells (10^5 or less). The mixture was incubated for 2 h at 37°C and then overnight at 4°C. At least 100 rosettes per sample were counted in a hemocytometer. The number of hapten-specific RFC was calculated from the number of cells forming rosettes with hapen-coupled SRBC minus the number of SRBC-RFC.

Radioautography. $5 \times 10^5 - 2 \times 10^6$ cells were suspended in 0.2 ml HEM 10% FCS, 0.5 μg ^{125}I-labeled anti-DNP Ig was added and the samples kept on ice for 30 min. The cells were washed twice through FCS underlayers, smeared and processed for radioautography as previously described (24). The smears were exposed for 3 days. Cells with more than 20 grains were counted as positive and at least 10^3 cells were scored per smear. Iodinations were performed by the chloramine-T method giving a substitution rate of less than one ^{125}I atom per molecule antibody.

Results

Binding of Normal Mouse Lymphocytes to DNP-Gelatin-coated Dishes. The proportion of spleen cells which bound to dishes coated with DNP-gelatin of various substitution ratios was determined in several experiments. The proportion of spleen cells binding to unconjugated gelatin layers varied from experiment to experiment; however, it never exceeded 0.25%. DNP_4-gelatin adsorbed 0.4% normal spleen cells. Increasing numbers of cells were bound with increasing substitution ratios of the DNP-gelatin adsorbents (Fig. 1). More than 2% of cells were bound to DNP_{16}-gelatin.

The number of binding cells reached a maximum after 60 min of fractionation and remained constant when the fractionation time was extended up to 3 h (Fig. 2). This was the case for both highly substituted DNP-gelatin which bound 3% after 60 min and lightly substituted DNP-gelatin which bound 1% in the same time. Thus, not the area of the dish but the number of binding cells was limiting,

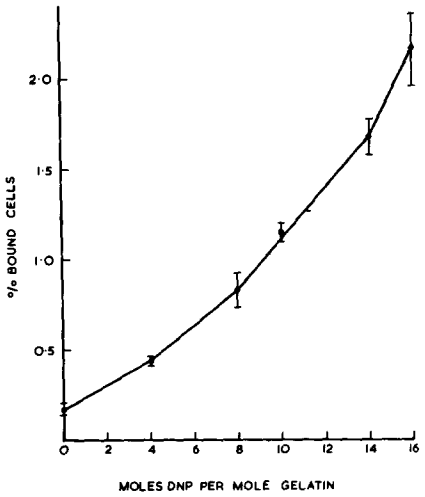

FIG. 1. Binding of normal spleen cells to dishes coated with different DNP-gelatin conjugates. Normal spleen cells were fractionated for 60 min in DNP-gelatin-coated dishes. Each point represents the percentage of cells binding to DNP-gelatin of the indicated substitution ratio (mean ± SE of three or more replicates). The data are pooled from four different experiments.

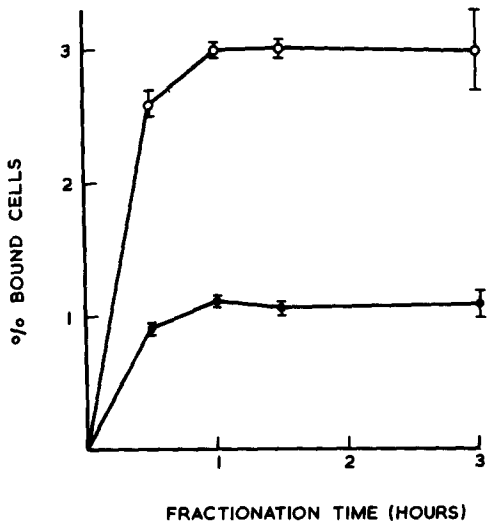

FIG. 2. Kinetics of the binding of normal spleen cells to DNP-gelatin coated dishes. Normal spleen cells were fractionated for 30–180 min in dishes coated with DNP_{19}-gelatin (○, 10^7 cells per dish) or coated with a mixture of equal volumes of DNP_{10}-gelatin and gelatin (●, 3×10^7 cells per dish). The figures represent the mean of triplicates ± SE of the percentage of binding cells.

suggesting that only defined subpopulations of spleen cells were adsorbed to each type of dish. Most of the DNP-gelatin-binding cells were B cells. 92% of DNP_8-gelatin-binding cells and 41% unfractionated cells stained with fluorescein-labeled antimouse Ig. Thymocytes did not bind at all to DNP-gelatin layers.

Inhibition of the Binding of Spleen Cells to Hapten-gelatin Layers. Fractionation of normal spleen cells in DNP_8-gelatin-coated dishes (5×10^7 cells per dish) in the presence of a polyvalent rabbit antimouse Ig (0.4 mg/ml) resulted in an almost complete inhibition of binding. Pretreatment of the cells with antimouse Ig at 37°C for 15 min inhibited also 94% of the binding.

Furthermore the binding of spleen cells to DNP_4-gelatin layers was inhibited in the presence of DNP-lysine up to 90% and in the presence of DNP-gelatin or DNP-BSA up to 100%. When the inhibitor concentration was expressed in equivalent molar concentrations of DNP (Fig. 3), it was obvious that the hapten was more effective as an inhibitor when present in a multivalent form than when present as monovalent DNP-lysine. Higher conjugated DNP_{24}-gelatin and

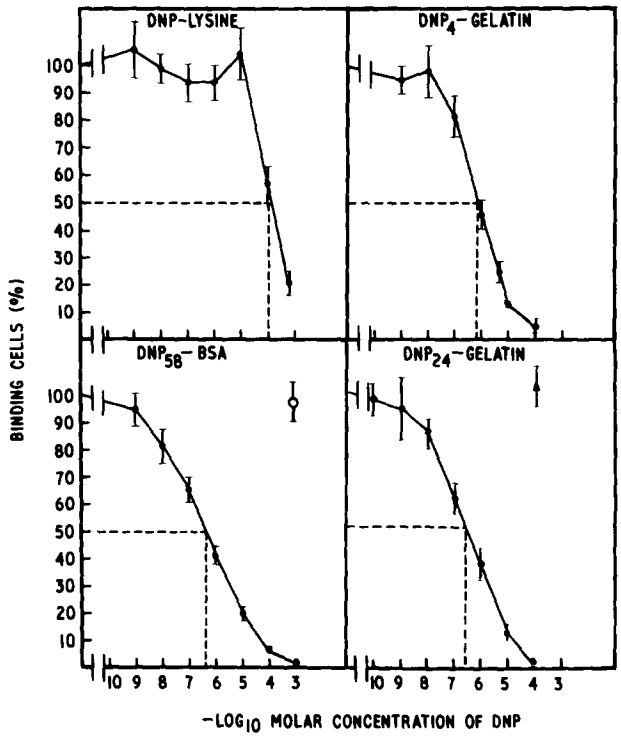

FIG. 3. Inhibition of binding of spleen cells to DNP-gelatin by free hapten or antigen. Normal spleen cells were fractionated in DNP_4-gelatin coated dishes in the absence or presence of different concentrations of DNP-lysine, DNP_4-gelatin, DNP_{24}-gelatin or DNP_{58}-BSA. The figures show the number of binding cells expressed as a percentage of the number of binding cells in the absence of inhibitor (mean of triplicate ± SE). The inhibitor concentrations are expressed as $-\log_{10}$ of molar concentrations of DNP-lysine or equivalent molar concentrations of DNP when DNP-protein conjugates were used as inhibitors. The binding of spleen cells to DNP_4-gelatin was not inhibited by gelatin (▲) or BSA (○) in concentrations corresponding to the highest concentration used with the DNP- conjugates.

DNP_{58}-BSA inhibited at slightly lower equivalent molar concentrations of DNP than DNP_4-gelatin. However, there was no significant difference in the inhibitory capacity of DNP_{24}-gelatin and DNP_{58}-BSA. Soluble gelatin or BSA, even in high concentrations, did not affect the binding to DNP_4-gelatin. Similarly the binding to NIP-gelatin-coated dishes was not significantly altered in the presence of soluble gelatin (Table I). However, soluble homologous hapten-gelatin inhibited almost completely and heterologous hapten-gelatin inhibited partially the binding of normal spleen cells to hapten-gelatin (Table I).

Antigen Binding Capacity of Purified Hapten Specific Lymphocytes. DNP-gelatin could not be washed off from the surface of all cells which were recovered from DNP-gelatin layers by melting. 15% of spleen cells recovered from DNP_8-gelatin layers bound ^{125}I-labeled MOPC 315 anti-DNP Ig as detected by radioautography whereas unfractionated control cells remained unlabeled.

TABLE I

Inhibition of Binding of Normal Spleen Cells to Dishes Coated with DNP-Gelatin or NIP-Gelatin by Soluble DNP-Gelatin and NIP-Gelatin

Inhibitor (0.5 mg/ml)	No. of binding cells ($\times 10^{-3}$)	
	DNP_4-gelatin dishes	NIP_3-gelatin dishes
None	160.4 ± 2.6	19.4 ± 3.2
Gelatin	153.8 ± 18.6 (4.1)	17.9 ± 3.8 (7.7)
DNP_4-gelatin	21.4 ± 2.2 (86.7)	15.5 ± 1.2 (20.1)
NIP_3-gelatin	138.7 ± 6.8 (13.5)	1.7 ± 0.7 (91.2)

Normal spleen cells were fractionated in DNP_4-gelatin and NIP_3-gelatin coated dishes (3×10^7 cells per dish) in the presence or absence of soluble gelatin, DNP_4-gelatin or NIP_3-gelatin (0.5 mg/ml). The number of binding cells per dish (mean of triplicates ± SE) is given with the percentage inhibition of binding in brackets.

In order to remove the DNP-gelatin which could not be washed off from the binding cell population, spleen cells recovered from DNP_8-gelatin-coated dishes were treated with collagenase for 30 min at 4°C or for 10 min at 37°C. The cells were then washed twice through underlayers of FCS and incubated at 4°C with ^{125}I-labeled anti-DNP Ig. No labeled cells were detected after treatment with collagenase although more than 10^4 cells were scored. Treatment with collagenase did not affect the cell viability and their ability to stain with a fluorescein-labeled polyvalent antimouse Ig: more than 90% of the cells were labeled before and after treatment.

Furthermore, the capacity of cells recovered from hapten-gelatin layers to bind hapten conjugated SRBC could be demonstrated after treatment with collagenase. DNP-gelatin binding cells did not form any DNP-specific rosettes unless treated with collagenase. However, after collagenase treatment, almost 100 times as many DNP-RFC were detected in DNP-gelatin binding cells as in unfractionated cells. The number of DNP-RFC was increased to the same degree in the unfractionated and purified cell population by using DNP-SRBC with a

higher surface density of DNP groups. Collagenase-treated NIP-gelatin-binding cells contained more than 200 times as many NIP-FRC as unfractionated spleen cells. Purified DNP- and NIP-specific spleen cells did not form SRBC-RFC (less than 0.01%), but the DNP-gelatin-binding cells were also slightly enriched in NIP-RFC and the NIP-gelatin binding cells in DNP-RFC (Table II).

Discussion

Gelatin forms an insoluble network at sufficiently high concentrations below a critical temperature. The melting point of a gelatin-gel is relatively sharp as is characteristic for network gels which are stabilized by secondary forces such as hydrogen bonds. The gelation and melting characteristics of gelatin are not significantly affected when small haptens are coupled to its lysine residues, which are not involved in the gelation process (25). Thus, hapten-coupled insoluble gelatin layers can be prepared readily and used as immunoadsorbents (18). The advantage of hapten-coupled gelatins as cellular immunoadsorbents is

TABLE II

Enrichment of Hapten-Specific RFC

Spleen cells	DNP-RFC	NIP-RFC
	%	%
Unfractionated	0.18 (0.31)	0.06
DNP_4-gelatin-binding cells	15.03 (42.10)	0.26
NIP_3-gelatin-binding cells	0.76	14.07

Unfractionated DNP_4-gelatin and NIP_3-gelatin binding spleen cells were treated with collagenase (10 min at 37°C). Then the cells were assayed for DNP- and NIP-RFC as described in Materials and Methods. In brackets are data obtained in a separate experiment using indicator SRBC with increased DNP density.

that specifically adsorbed cells can be recovered by melting the gel at physiological temperatures.

The proportion of normal spleen cells binding to hapten gelatin-coated dishes depended on the hapten and its density on the adsorbent. The number of binding cells reached a maximum after a sufficiently long fractionation time depending on the number of cells fractionated per dish. The fact that the number of binding cells did not increase continuously in dishes whose binding sites were not yet saturated suggested strongly that only a defined subpopulation of cells was bound to a particular type of adsorbent.

The binding of normal spleen cells to DNP-gelatin-coated dishes was inhibited by a polyvalent antimouse Ig and by free hapten. Thus the binding population was restricted to lymphocytes bearing DNP-specific surface receptors. There was no evidence for binding of lymphocytes to gelatin determinants or new antigenic determinants of DNP-gelatin conjugates, since the binding to DNP-gelatin-coated dishes could be inhibited by DNP-lysine, DNP-gelatin, and DNP-BSA but not by unconjugated gelatin or BSA. DNP-protein conjugates inhibited the binding of spleen cells to DNP-gelatin-coated dishes at lower equivalent molar

concentrations of DNP than DNP-lysine. The superiority of multivalent hapten conjugates over monovalent hapten in competitive inhibition of rosette formation with hapten-coupled SRBC has been demonstrated also by other authors (26). Although binding of spleen cells to DNP-gelatin-coated dishes was prevented in the presence of free hapten, the DNP-gelatin-bound cells were not eluted from the adsorbent by free DNP-lysine or DNP-gelatin.

Thus the binding of lymphocytes to hapten-gelatin layers is the result of a sequence of complex events. In the first step, simultaneous or almost simultaneous interactions between haptenic determinants and more than one cell surface receptor or one cell are presumably required to provide enough binding energy to retain the cell. In the second step further specific and nonspecific bonds together with changes of the cell shape lead to a highly multivalent binding which is essentially irreversible. Rosette formation of hapten-specific lymphocytes with hapten-coupled red cells represents a similar irreversible reaction as judged by the failure of an excess of free hapten, added after rosette formation to dissociate the rosettes (26).

The number of simultaneous or almost simultaneous interactions of a cell surface with the adsorbent required for the initial trapping should be lower for cells with high affinity for the hapten and higher for cells with lower affinity. Furthermore the probability that one cell binds simultaneously to more than one haptenic determinant increases with increasing density of the adsorbent. Thus highly conjugated DNP-gelatin layers should bind more cells than low conjugated DNP-gelatin due to additional binding of lower affinity cells. A related observation has been described again with hapten-specific RFC. More RFC with lower affinity receptors have been detected with indicator cells bearing a higher hapten density on their surface (26).

The presence of DNP-gelatin on the surface of isolated cells after fractionation is a disadvantage especially for quantitative antigen-binding studies with the purified cell population. However all specific cell separation methods involve essentially binding of antigenic determinants in a highly multivalent fashion which cannot be reversed readily. In the methods which do not use immunoadsorbents, binding of red cells (2–5) or fluorescein-labeled antigen (6, 7) is the essential prerequisite of the fractionation procedure. Cells eluted by mechanical means from plastic beads to which the antigen was attached noncovalently also carry antigen (10). Cells removed mechanically from nylon fibers to which the antigen was coupled covalently however lose their receptors on the adsorbent (15). In our experiments DNP-gelatin remained bound to the surface of a substantial number of binding cells after recovery by melting. However, it could be washed off after brief treatment of the isolated cells with collagenase at 37°C or at 4°C. Cell viability and surface receptors were not affected by the enzyme. The same proportion of cells were labeled with a fluoresceinated antimouse Ig before and after treatment with collagenase. Furthermore we were able to demonstrate the capacity of the isolated hapten-specific cells to rebind the specific antigen.

The majority of DNP_4-gelatin-binding cells appeared to be thymus-independent (B−) lymphocytes since more than 90% of these cells were stained with fluoresceinated antimouse Ig. Thymocytes did not bind at all to gelatin or

DNP-gelatin-coated dishes. So far we have not excluded specific binding of peripheral thymus-dependent (T−) lymphocytes to hapten-gelatin layers. Preliminary data show that some T lymphocytes were bound especially to highly substituted DNP-gelatin layers. However, the specificity and the function of the binding T lymphocytes remain to be determined. The isolated cells are highly viable and their specific functions can be tested in various systems. In the accompanying paper we describe the capacity of purified hapten-specific spleen cells from unimmunized mice to be stimulated in vitro to give rise to a specific antihapten antibody-forming cell response.

Summary

Normal mouse spleen cells were fractionated in dishes coated with thin layers of DNP-gelatin or NIP-gelatin, which were insoluble at 4°C. Highly viable cells were recovered from the dishes by melting the gel at 37°C.

NIP_3-gelatin layers bound approximately 0.1% and DNP_4-gelatin layers 0.5% of normal spleen cells. Increasing numbers of low affinity cells were bound with increasing DNP density of the adsorbent. The binding to insoluble DNP-gelatin was hapten-specific since it was inhibited by DNP-lysine, soluble DNP-gelatin or DNP-BSA but not by soluble gelatin or bovine serum albumin (BSA). It was also inhibited by a polyvalent rabbit antimouse Ig.

DNP-gelatin was detected on the surface of cells recovered from DNP-gelatin-coated dishes by ^{125}I-labeled anti-DNP Ig. The cell surface bound DNP-gelatin could be removed by treatment with collagenase. Collagenase treatment did not detectably affect cell viability or surface receptors. More than 90% of DNP-gelatin-binding cells were labeled with a polyvalent ^{125}I-labeled antimouse Ig before or after collagenase treatment under conditions known to label B lymphocytes. Furthermore, the specific antigen-binding capacity of the purified cell populations could be demonstrated after treatment with collagenase. Purified DNP_4-gelatin binding cells contained more than 100 times as many DNP-RFC than unfractionated cells. The enrichment of NIP-RFC in the cell population recovered from NIP_3-gelatin-coated dishes was more than 200-fold.

We thank Dr. Kincade for supplying purified antimouse Ig, FITC-antimouse Ig, and MOPC 315 anti-DNP Ig. We also thank Mr. J. Pye for performing the iodinations and Mrs. K. Fowler for technical assistance. We are grateful to Professor G. J. V. Nossal and Dr. A. Szenberg for advice and encouragement throughout this work.

Received for publication 29 October 1974.

References

1. Shortman, K. 1974. Separation methods for lymphocyte populations. *In* Contemporary Topics in Molecular Immunology. G. A. Ada, editor. **11**:161.
2. Osoba, D. 1970. Some physical and radiobiological properties of immunologically reactive mouse spleen cells. *J. Exp. Med.* **132**:368.
3. Wilson, J. D. 1973. The function of immune T and B rosette forming cells. *Immunology.* **25**:185.
4. Crone, M., K. Hala, and M. Simonsen. 1969. Rosette forming cells in the chicken. Purification and the testing of other properties. *In* Developmental Aspects of

Antibody Formation and Structure. J. Sterzl and H. Riha, editors. Academia, Prague, **1**:155.

5. Brody, T. 1970. Identification of two cell populations required for mouse immunocompetence. *J. Immunol.* **105**:126.
6. Julius, M. H., T. Masuda, and L. A. Herzenberg. 1972. Demonstration that antigen binding cells are precursors of antibody producing cells after purification with a fluoresceing activated cell sorter. *Proc. Natl. Acad. Sci. U.S.A.* **69**:1934.
7. Julius, M. H., and L. A. Herzenberg. 1974. Isolation of antigen-binding cells from unprimed mice. Demonstration of antibody-forming cell precursor activity and correlation between precursors and antibody avidities. *J. Exp. Med.* **140**:904.
8. Wigzell, H., and B. Andersson. 1969. Cell separation on antigen-coated columns. Elimination of high rate antibody-forming cells and immunological memory cells. *J. Exp. Med.* **129**:23.
9. Wigzell, H. 1970. Specific fractionation of immunocompetent cells. *Transplant. Rev.* **5**:76.
10. Moroz, L. A., and A. O. Kotoulas. 1973. Cell fractionation on affinity columns. *In vivo* function of hemocyanin-binding lymphocytes from normal mouse spleen. *Inst. Arch. Allergy.* **44**:657.
11. Truffa-Bachi, P., and L. Wofsy. 1970. Specific separation of cells on affinity columns. *Proc. Natl. Acad. Sci. U.S.A.* **66**:685.
12. Davie, J. M., and W. E. Paul. 1971. Receptors on immunocompetent cells. II. Specificity and nature of receptors on [^{125}I]DNP-GPA binding lymphocytes of normal guinea pigs. *J. Exp. Med.* **134**:495.
13. Rubin, B., and H. Wigzell. 1973. The immune response against hapten autologous protein conjugates in the mouse. III. Specificity of co-operating non-thymus-processed (B) and thymus-processed (T) lymphocytes. *J. Exp. Med.* **137**:911.
14. Schlossman, S. F., and L. Hudson. 1973. Specific purification of lymphocytes populations on a digestible immunoadsorbent. *J. Immunol.* **110**:313.
15. Edelman, G. M., U. Rutishauser, and C. F. Millette. 1971. Cell fractionation and arrangement on fibres, beads and surfaces. *Proc. Natl. Acad. Sci. U.S.A.* **68**:2153.
16. Choi, T. K., D. R. Sleight, and A. Nisonoff. 1974. General method for isolation and recovery of B cells bearing specific receptors. *J. Exp. Med.* **139**:761.
17. Selective deletion of lymphocytes. Brook Lodge Symposium on Immunologic Intervention. J. W. Uhr and M. Landy, editors. Academic Press Inc., New York. 53.
18. Haas, W., J. W. Schrader, and A. Szenberg. 1974. A new, simple method for the preparation of lymphocytes bearing specific receptors. *Eur. J. Immunol.* **4**:565.
19. Eisen, H. N. 1964. Preparation of purified anti. 2, 4-dinitrophenyl antibodies. *In* Methods in Medical Research. H. N. Eisen, editor. Year Book Medical Publishers Inc., Chicago. **10**:94.
20. Brownstone, A., N. A. Mitchison, and R. Pitt-Rivers. 1966. Chemical and serological studies with an iodine-containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) and related compounds. *Immunology.* **10**:465.
21. von Boehmer, H., and K. Shortman. 1973. The separation of different cell classes from lymphoid organs. IX. A simple and rapid method for removal of damaged cells from lymphoid cell suspension. *J. Immunol. Methods.* **2**:293.
22. Kincade, P. W., and M. D. Cooper. 1971. Development and distribution of immunoglobulin-containing cells in the chicken. An immunofluorescent analysis using purified antibodies to μ, γ and light chains. *J. Immunol.* **106**:371.
23. Feldmann, M., and A. Basten. 1971. The relationship between antigen structure and the requirement for thymus-derived cells in the immune response. *J. Exp. Med.* **134**:103.

24. Nossal, G. J. V., N. L. Warner, H. Lewis, and J. Sprent. 1972. Quantitative features of a sandwich radioimmunolabeling technique for lymphocyte surface receptors. *J. Exp. Med.* **135**:405.
25. Grabar, P., and J. Morel. 1950. Sur la participation du groupement guanidique de l'arginine à la gèlification de la gèlatine. *Bull. Soc. Chem. Biol.* **32**:643.
26. Möller, E. 1974. Specificity of hapten-reactive T and B mouse lymphocytes. Affinity and avidity of T- and B-cell receptors and antihapten antibodies as factors of dose and time after immunization. *Scand. J. Immunol.* **3**:339.