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Demonstration That Antigen-Binding Cells Are Precursors of Antibody-Producing Cells After Purification with a Fluorescence-Activated Cell Sorter

(keyhole-limpet hemocyanin/human-serum albumin/adoptive secondary response/ lymphocyte receptors/thymus-derived, bone marrow-derived cells)

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ABSTRACT **We have obtained viable and functional populations of antigen-binding cells enriched up to 500-fold from primed spleen-cell suspensions by fluorescent labeling and by a new electronic cell sorter that sorts viable cells according to fluorescence. Concomitantly, populations largely depleted of antigen-binding cells were obtained. While neither population alone is capable of a full adoptive secondary response when injected into irradiated recipients, a reconstituted mixture restores the full response of the unfractionated spleen cells. Admixture of sources of unprimed thymus-derived cells (T-cells) with the purified antigen-binding cells (B-cells) restores much of the full response.**

Cooperation between two types of lymphocytes is required for the production of antibodies to such antigens as heterologous erythrocytes (1), proteins (2), and haptens conjugated to these antigens (3). Adoptive primary or secondary antibody responses are severely depressed in irradiated recipients of lymphoid cells that are depleted for thymus-derived (T) cells by treatment with antibodies to the theta antigen, but that can be restored with primed or unprimed T-cells (4). Conversely, depletion of specific antigen-binding cells with antigen-conjugated columns (5) or by "hot antigen suicide" (6) also depresses adoptive antibody responses.

Enriched suspensions of specific antigen-binding cells, which are functionally precursors of active antibody-forming cells or cooperators in development of active antibody-forming cells would be useful in further characterization of cellular events involved in antibody formation. It has been reported that passage of lymphoid cells through antigen-conjugated columns and subsequent elution of the bound cells [by use of a hapten system, lactose–keyhole limpet hemocyanin (lactose-KLH)], yields an immunocompetent population that restores the response to lactose in irradiated lactose–KLH primed animals (7). We have labeled, by immunofluorescence, antigen-binding cells in spleen cell suspensions, either directly with fluorescein-conjugated antigen or indirectly with fluorescein-conjugated antibody to the antigen-coated cells, and then isolated these labeled cells by passage of the suspension through an electronic, fluorescence-activated cell sorter (8).

In this publication, we present evidence showing that antigen-binding cells isolated in this fashion are competent to produce an adoptive secondary immune response, with KLH and human serum albumin (H-albumin) as antigens. We show that the response of the KLH-binding cells is dependent upon the addition of T-cells, indicating that the antigen-binding cells are bone marrow-derived (B) cells, and therefore are the precursors of the antibody-forming cells (1).

Abbreviations: KLH, keyhole limpet hemocyanin; T-cell, thymus-derived cell; B-cell, bone marrow-derived cell; H-albumin, human serum albumin.

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MATERIALS AND METHODS

Mice. Two pairs of strains of congenic mice were used in these experiments. CWB/13 (Ig^b) (9) is the congenic partner to CSW (C3H.SW/SnHz) (Ig^a) and BAB/14 (Ig^b) (kindly supplied by Dr. M. Potter and maintained in this laboratory) is the congenic partner to BALB/cN (Ig^b).

Immunizations. CWB mice each received 100 μg of H-albumin in complete Freund's adjuvant in each hind footpad. BALB/cN mice each received 400 μg of heat-aggregated human gammaglobulin (10), intravenously. BALB/cN and BAB/14 mice each received 100 μg of aqueous KLH, intravenously.

Cell Suspension, Cell Transfers, Irradiation, and Collection of Serum were all described (4), except that cells were filtered through glass wool at 22–25°. Less than 1% of morphologically detectable macrophages remained. Dulbecco's phosphate-buffered saline (pH 7.4) (11) containing 5% fetal-calf serum was used as diluent for all reagents used with cells.

Immunofluorescent Staining. Spleen cells bound to KLH were stained directly with fluorescein-conjugated KLH (0.5 mg/ml per 10^7 cells). Spleen cells bound to H-albumin or H-gammaglobulin were stained by indirect techniques with 0.8 mg/ml of H-albumin per 2×10^7 cells or 0.4 mg/ml of H-gammaglobulin per 10^7 cells for the first layers, respectively. After washing, fluorescein-conjugated anti H-albumin from rabbit (4 mg/ml per 4×10^7 cells) or anti H-gammaglobulin from goat (2 mg/ml per 10^7 cells) were used for the second layers. Incubations were at 22–25° for 15 min., washes were through gradients of fetal-calf serum, and fluorescein-conjugated proteins had 1–3 mol of fluorescein per 100,000 daltons of protein.

Fluorescence Microscopy. A Zeiss microscope with an HBO, 200 mercury arc (OSRAM) light source was used. A combination of Zeiss BG3 excitation and No. 47 barrier filters were used to detect fluorescence. Cells exhibiting speckled, ringed, or crescent (cap-like) fluorescence were considered positive.

TABLE 1. *Enrichment of antigen-binding cells*

Exp.	Strain	Priming antigen	Staining technique	% Labeled cells: Before separation	% Labeled cells: Undeflected	% Labeled cells: Deflected	Enrichment factor
1	BALB/cN*	KLH	Direct	0.1†	<0.1	40	400
2	BAB/14	KLH	Direct	0.1	<0.1	52	520
3‡	CWB	H-albumin	Indirect	3.5	1	65	18.5
4	CWB	H-albumin	Indirect	1.3	<0.1	55	42
5	BALB/cN	Human gammaglobulin	Indirect	1	<0.1	62	62

* Animals were primed with the specified antigen (see *Methods*) for 2–3.5 months.

† The percentage of KLH-binding cells varied with the batch of fluoresceinated KLH used.

‡ In Exp. 3–5, separation was by a double-pass procedure in which cells from the first deflected and undeflected samples were concentrated by centrifugation (if necessary) and subjected to a second separation. This permits separation of a larger number of cells in a shorter time with somewhat greater purity.

Cell Separations. Our present instrumentation (8) enables separation of cells binding a detectable quantity (about 1 to 2 $\times$ 10^4 fluorescein residues/cell) of fluorescein-conjugated protein. Cells in liquid suspension are forced under pressure through a micronozzle that produces an effluent jet 50 μm in diameter. The architecture of this nozzle creates a coaxial flow system whereby cells injected along the center line are confined to the central 12 μm of the effluent jet. The nozzle is vibrated axially at 4 $\times$ 10^4 cycles/sec, breaking the jet into 4 $\times$ 10^4 uniform droplets/sec. Immediately below the nozzle, before droplet formation occurs, the jet passes through a focused argon ion laser beam that excites fluorescence on those cells bearing fluorescein-conjugated protein. Fluorescent light from such cells is imaged on a photomultiplier tube with dark-field optics and the appropriate filters to exclude scattered exciting light.

When a cell-derived voltage pulse from the photomultiplier exceeds a pre-set threshold, the effluent jet is charged electrically. The charging signal is timed so that the detected cell will be trapped in a charged droplet formed during the charging interval. Droplets formed in the absence of a charging voltage will be uncharged.

The stream, subsequent to droplet formation, passes through an electrostatic field, which causes deflection of charged droplets into one container, while uncharged droplets remain undeflected and collect in another container. The cells are maintained at 4° throughout the separation except for a few seconds during actual deflection.

Titrations. All sera from the adoptive transfers to KLH were individually titrated by the passive microhemagglutination technique (12). KLH was coupled to sheep erythrocytes by a modification of the glutaraldehyde method (13). Sera were titrated after reduction with 0.01 M dithioerythritol; equal volumes of sera and dithioerythritol were incubated at 37° for 30 min.

Sera from mice in which adoptive transfers to H-albumin were made, were titrated by a modified Farr precipitation technique (14). Each assay tube contained 7.5 ng of ^{125}I-labeled H-albumin and 5 μl of serum dilution in a total volume of 100 μl of borate-buffered saline (pH 8.4) containing 10% normal mouse serum as diluent.

RESULTS

Separations of Antigen-Binding Cells. Table 1 shows data from several typical separations performed with the fluorescent-cell sorter. Antigen-binding cells in the spleens of several mouse strains primed for three antigens were stained by either a direct or indirect immunofluorescent technique. The number of stained cells in the original suspensions of spleen cells depends on the immunizing antigen, time after immunization, mouse strain, concentrations of antigen, concentration of fluorescent antibody, and whether direct or indirect staining is used. Nevertheless, we obtain marked enrichment of stained cells in deflected fractions and marked depletion of stained cells in undeflected fractions.

In experiments 1 and 2, a single pass of the stained spleen cells (containing 0.1% fluorescent cells) resulted in deflected fractions containing 40–52% fluorescent cells, representing a 400- to 500-fold enrichment. The double-pass procedure used in experiments 3, 4, and 5 (Fig. 1) yielded a deflected fraction containing an even larger proportion of fluorescent cells. Enrichment factors in experiments 3, 4, and 5 are considerably lower than in experiments 1 and 2, because the former contained a 10-fold greater proportion of fluorescent cells in the starting population (before separation). The double-pass technique is more efficient in that it allows separation of a greater number of cells in a shorter period of time, resulting in purity of the deflected fractions equal to or greater than that derived from a single pass at a lower starting cell concentration (see Fig. 1).

Adoptive Response to KLH. The adoptive secondary response of irradiated (600 R) BALB/cN mice that received graded doses of KLH-primed spleen cells is presented in Table 2. Dithioerythritol-resistant anti-KLH response is measured by passive hemagglutination (12).

Table 2 shows that an adequate response (titer of 12 $\pm$ 0.3) was obtained on transfer of as few as 1 $\times$ 10^6 primed

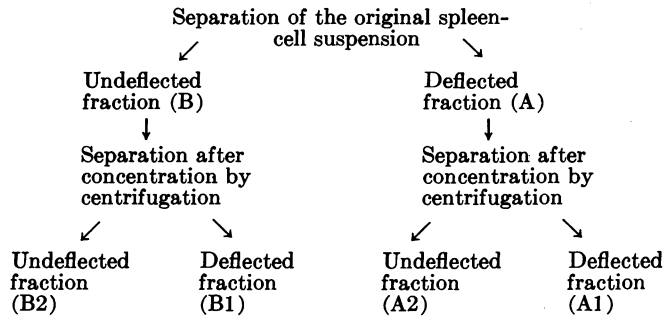

FIG. 1. Flow diagram for the double-pass procedure.

TABLE 2. *Dependence of the adoptive secondary response to KLH on the number of cells transferred*

Spleen donor	Number of cells transferred‡ ($\times 10^6$)	Anti-KLH titers,* ±SE ($\log_2$)	
		Unstained	
Unprimed	20	2.6 ± 0.5†	
	5	1.3 ± 0.3	
	0	1.0 ± 0	
Primed with KLH*	20	14 ± 0.5	
	5	14 ± 0.6	
	2	13 ± 0.5	
	1	12 ± 0.3	
	0.5	3.0 ± 1.0	
		Stained with fluorescent-KLH	
		Not passed through separator	Passed
	3	12 ± 0.3	11 ± 0.8

* Resistant to dithioerythritol at day 15 after transfer.

† Spleen cells were transferred into irradiated syngeneic hosts along with 3 × 10^6 syngeneic bone marrow cells and 100 μg of KLH.

‡ Each number represents the mean titer of sera from three animals.

spleen cells. If fewer than 1 × 10^6 primed spleen cells are injected, the response drops off sharply (3.0 ± 1.0). A 20-fold increase in the number of injected primed cells (2 × 10^7) only slightly raised the resulting titer to 14 ± 0.5. The same number (2 × 10^7) of virgin spleen cells gave a much lower titer of 2.6 ± 0.5.

Fluorescent staining and passage of primed spleen cells through the cell sorter did not significantly decrease their ability to transfer a response (see Table 2).

To demonstrate that separation of antigen-binding cells depletes the immune response of whole spleen, the original number of unseparated transferred cells must be close to limiting. Therefore, we used 3 × 10^6 primed spleen cells in subsequent transfer experiments. This number of cells contained about 3 × 10^4 stained cells.

The first experiment in Table 3 shows that depletion of KLH-binding cells significantly diminished the ability of 3 × 10^6 complementary undeflected (non-KLH-binding) spleen cells to transfer a secondary response.

In the same experiment, 3 × 10^4 cells from the deflected population, containing 38% KLH-binding cells, responded to KLH on transfer, although the response was significantly lower than that of 3 × 10^6 unseparated spleen cells.

The second experiment confirmed the decreased response of 3 × 10^6 undeflected cells, but in this case, the response of 3 × 10^4 deflected cells, containing 61% KLH-binding cells was just detectable. Similarly, in the third and fourth experiments, 5 × 10^4 deflected cells, containing 65 and 70% KLH-binding cells, respectively, gave a barely detectable response. Thus, as the purity of the deflected fractions increased, their ability to give a response on transfer decreased.

Addition of 3 × 10^6 undeflected cells to 3 or 5 × 10^4 deflected cells restored the response to the level given by 3 × 10^6 unseparated cells. Addition of 10-fold fewer undeflected cells proved inadequate in restoring the response of the deflected fraction. Thus, there is a requirement for two cell types in the adoptive response to KLH, one of which is in the deflected fraction and the other in the undeflected fraction.

Adoptive Response to H-albumin. The results of separations and transfers of cells that bind H-albumin (in CWB mice) were essentially the same as those with KLH, although the number of cells required to transfer the maximum response was 10-fold greater. Recipients of either deflected or undeflected populations responded poorly to the albumin, compared to recipients of equivalent numbers of cells from whole primed spleen. However, injection of a mixture of deflected and undeflected cells, which reconstituted the original spleen-cell suspension, restored the anti-H-albumin response to the titer achieved with intact spleen.

In Table 4 the pooled results of three H-albumin transfer experiments are presented. Data for anti-H-albumin response is presented as units (see† footnote, Table 4) of antibody activity per μl of serum [1 unit of antibody activity equals the amount of activity necessary to precipitate 33% of ^{125}I-labeled H-albumin by the Farr technique (see *Methods*)].

Recipients of 10^7 unseparated cells, containing (1.5–2) × 10^5 stained cells, had an average titer of 3.9 units of anti-H-albumin activity per μl serum. Recipients of 4 × 10^5 deflected cells, containing (1–2) × 10^5 stained cells, showed an average response of 0.6 units of anti-H-albumin activity per μl of serum. Animals receiving 10^7 undeflected cells alone (containing less than 10^4 stained cells) also responded significantly, although poorly, showing 0.4 units of anti-H-albumin activity per μl of serum. However, recipients of 5-fold fewer undeflected cells failed to make a detectable response.

In contrast, recipients of a mixture of 10^7 undeflected plus 4 × 10^5 deflected cells responded as well as recipients of 10^7 whole spleen cells. Thus, as already demonstrated in the KLH system, there is a requirement for two cell types in the adoptive response to H-albumin, one of which is in the deflected fraction and the other in the undeflected fraction.

Requirement for T-cells in the Adoptive Response to KLH. Evidence that the deflected fractions contained precursors of KLH antibody-forming cells (B-cells) is presented in experiments 3 and 4, Table 3, where addition of a source of virgin T-cells to the deflected fraction restored its capacity to produce antibody in an adoptive response. In these experiments, neither 1 × 10^6 virgin spleen cells nor 70 or 90 × 10^6 thymus cells alone, gave an adoptive response. However, when injected together with 5 × 10^4 deflected cells, both the virgin spleen and virgin thymus cells cooperated with the deflected antigen-binding cells to increase the anti-KLH titer.

Since virgin T-cells can replace the undeflected fraction, this fraction must contain cooperating T-cells. The titers on addition of either thymus or spleen, however, were lower than the titers given by the equivalent mixture of deflected and undeflected cells, suggesting that the cooperators in the undeflected fraction of primed spleen were more efficient than those in virgin spleen or thymus.

DISCUSSION

The data presented in this publication demonstrate directly that the precursor cells of antibody-forming cells, at least in a secondary response, have receptor sites that bind antigen. Starting with cells from the spleen of a KLH- or H-albumin primed animal, we have made cells that bind the antigen visible with fluorescent staining, and separated them from the

TABLE 3. *Adoptive response after separation of KLH antigen-binding cells*

Exp.	% Fluorescent cells: Unseparated	% Fluorescent cells: Deflected	% Fluorescent cells: Undeflected	Number of cells transferred ($\times 10^6$)*: Unseparated	Number of cells transferred ($\times 10^6$)*: Deflected	Number of cells transferred ($\times 10^6$)*: Undeflected	Unprimed: Spleen	Unprimed: Thymus	Anti-KLH† titer $\log_2$
1	1.3	38	<0.1	3	—	—	—	—	10 ± 0.8
				—	—	3	—	—	6.7 ± 0.8
				—	0.03	—	—	—	5.0 ± 1.5
2	1.0	61	<0.1	3	—	—	—	—	7.6 ± 0.3
				—	—	3	—	—	2.7 ± 0.8
				—	—	0.3	—	—	1.6 ± 0.6
				—	—	0.1	—	—	<1
				—	0.03	—	—	—	<1
				—	0.03	3	—	—	6.3 ± 0.6
				—	0.03	0.3	—	—	3.0 ± 2.0
				—	0.03	0.1	—	—	1.5 ± 0.5
3	1.3	65	<0.1	3	—	—	—	—	7.3 ± 0.3
				—	—	3	—	—	2.0 ± 0
				—	0.05	—	—	—	1.5 ± 0.5
				—	0.05	3	—	—	7.0 ± 0
				—	—	—	1	—	<1
				—	—	—	—	70	<1
				—	0.05	—	1	—	4.5 ± 0.1
				—	0.05	—	—	70	4.3 ± 0.8
4	1.2	70	<0.2	3	—	—	—	—	4.6 ± 0.3
				—	—	3	—	—	1.0 ± 0
				—	0.05	—	—	—	1.3 ± 0.3
				—	0.05	3	—	—	3.6 ± 0.3
				—	—	—	1	—	<1
				—	—	—	—	90	<1
				—	0.05	—	1	—	2.8 ± 0.3
				—	0.05	—	—	90	2.3 ± 0.3

* Spleen cells from animals primed 2–3.5 months earlier were transferred into irradiated syngeneic or congenic hosts along with 3×10^6 syngeneic bone marrow cells and 100 μg of KLH.

† $\log_2$ anti-KLH titers (0.01 M dithioerythritol-resistant) ±SE at day 15 after administration of antigen. Each number represents the mean titer of sera from three animals.

remainder of the cell population by passage through a fluorescence-activated electronic cell separator. The separated cells, when transferred into irradiated hosts were unable to respond to antigen to give a normal adoptive secondary response. However, supplementation with cells from the undeflected (unstained) fraction completely restored the response. We have not examined in either of the transfer systems used, to what extent the separated antigen-binding cells, when supplemented with the undeflected fraction, might also restore the response to an unrelated antigen.

Substitution of unprimed thymocytes or spleen cells for cells from the undeflected fraction, in the KLH experiments, also restored the adoptive response although less efficiently. Thus, we conclude that the deflected (antigen-binding) cells

TABLE 4. *Adoptive response after separation of H-albumin-binding cells*

% Fluorescent cells: Unseparated	% Fluorescent cells: Deflected	% Fluorescent cells: Undeflected	Number of cells transferred ($\times 10^6$)*: Unseparated	Number of cells transferred ($\times 10^6$)*: Deflected	Number of cells transferred ($\times 10^6$)*: Undeflected	Units of anti-H-albumin activity per μl of serum†
1–2	25–50‡	<0.1	10	—	— [8]§	3.9 (2.4–6.7)¶
			—	—	10 [8]	0.4 (0.3–0.7)
			—	—	2 [5]	<0.1
			—	0.4	— [12]	0.6 (0.2–1.5)
			—	0.4	10 [5]	8.9 (4.2–20)

* Primed CWB spleen cells were transferred into irradiated CSW hosts with 3×10^6 CWB bone marrow cells and 200 μg of alum-precipitated H-albumin.

† 1 unit of anti-H-albumin activity equals the amount of activity necessary to precipitate 33% of 7.5 ng of ^{125}I-labeled H-albumin by the Farr technique (see *Methods*). Anti-H-albumin activity was measured at day 14 after transfer.

‡ Data pooled from three experiments in which the deflected fractions contained 25, 42, or 50% fluorescent cells.

§ In brackets are numbers of mice per group.

¶ Geometric mean (±SE).

are bone marrow-derived (B-cell) precursors of antibody-forming cells and that the undeflected cells are a source of thymus-derived (T-cell) cooperators.

As the data from the KLH separation experiments show, increased efficiency of separation resulted in both a decreased ability of the deflected fraction to respond without the addition of cooperating T-cells and a decreased ability of the undeflected fraction to respond without the addition of B-cells. In the first experiment (Table 3) the deflected fraction, obtained from a single pass separation, contained only 38% fluorescein-labeled (antigen-binding) cells. Injection of 3×10^4 cells from this fraction gave a weak, although significant, adoptive response. In subsequent experiments the deflected fractions resulting from a double pass separation contained greater than 65% fluorescent labeled cells and the complementary undeflected fractions contained less than 0.1% fluorescein-labeled cells. This increase in the efficiency of separation rendered the separated fractions incapable of responding above the threshold of detectability.

Supplementation of the twice-passed deflected fractions with either undeflected cells or virgin T-cells restored the ability of the deflected fraction to respond to antigenic stimulation. The undeflected cells (derived from primed spleen) were the most efficient; 3×10^6 cells were able to restore the maximum response. Addition of either 1×10^6 virgin spleen, or 70 or 90×10^6 virgin thymus cells brought the response to two-thirds of maximum. It is not surprising that greater restoration of the response is accomplished when the deflected fraction is supplemented with undeflected fractions of primed spleen as opposed to virgin spleen or thymus cells, since interaction between primed B-cells (memory cells) and primed cooperators is known to be more efficient than that between primed B-cells and unprimed cooperating cell populations (15). Whether the maximum potential of the deflected cells could be obtained by addition of larger numbers of virgin T-cells was not investigated.

We have no explanation for the differences in titers of antibody to KLH obtained on transfer of unfractionated spleen cells in the experiments reported (Tables 2 and 3). In all the experiments, animals were primed 2–3.5 months before they were used as spleen cell donors. Further, a standard anti-KLH titrated as a control in each experiment did not vary in titer by more than one $\log_2$.

The use of primed animals as a source of cells has greatly facilitated the development of the separation methodology described here. The adoptive primary response to KLH, as seen in Table 2, is more difficult to work with due to the low titers elicited on transfer of relatively large numbers of cells. This is consistent with the fact that the number of detectable specific antigen-binding cells in a virgin (unprimed) spleen is 10- to 100-fold lower compared to the number in primed spleen (16). Also, there are either more cooperating T-cells in a primed animal or the cooperators are more efficient. Nonetheless, our separation technique is specifically designed to detect and separate rare cells, and we ultimately hope to investigate the primary response by use of a virgin spleen-cell population enriched with antigen-binding cells.

Our data should not be taken as indication that T-cells do not bind antigen, but merely that there is not enough antigen bound to T-cells to allow their deflection with the current sensitivity of the machine. T-cell cooperating activity is not destroyed by staining or machine passage as there are many active cooperators in the undeflected fraction. Therefore, the amount of antigen bound to cooperators (if any) is very small compared to the amount bound to precursors of antibody-forming cells.

The minimum threshold for detection on the cell separator is currently in the range of 1 to 2×10^4 residues of protein-bound fluorescein per cell, which is equivalent [with an F/P (fluorescein residues per mole of protein) of about 2] to 10^4 molecules of cell-bound KLH. Thus, from a spleen primed with KLH, T-cells must express fewer than 10^4 binding sites per cell, and B-cells greater than that number. Many of the cells exhibited considerably more fluorescence than the minimum detectable.

If we compare the KLH and H-albumin systems, it is interesting to note that it takes roughly 10 times as many cells from intact spleens primed with the H-albumin to maximally transfer the secondary response to the same antigen. The reasons for this are unclear, but do not appear to be due to fewer H-albumin-primed B-cells, since the percentage of antigen-binding (stained) cells in primed spleens is essentially the same for both antigens.

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