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Isolation of antigen-binding cells from unprimed mice

II. Evidence for monospecificity of antigen-binding cells*

Spleen cells from unimmunized mice were exposed to two contrastingly fluorescent antigens simultaneously. Antigen-binding cells of either specificity were isolated using a fluorescence-activated cell sorter (FACS). Purified cells binding one or the other of the antigens were then examined by fluorescence microscopy for the presence of bound antigen of the alternate specificity.

No double binding cells were seen. If cells bear receptors of two or more specificities and these receptors are randomly distributed among antigen-binding cells, then of the 13 000 binding cells examined 82 were expected to bind both antigens. These results strongly suggest that antigen-binding cells bear receptors of only one specificity. In addition, by inference from the functional correlation between antigen-binding cells and precursor cells, the data support the contention that precursors of antibody-forming cells are monospecific.

1. Introduction

A number of studies have provided generally accepted indirect evidence that antigen-binding cells contain the precursors of antibody-forming cells for the antigen studied [1, 2, 3]. Direct evidence has been found more recently using a fluorescence-activated cell sorter (FACS) [4] to isolate antigen-binding cells and directly demonstrate that they contain the precursor activity for the same antigen specificity [5]. However, for most antigens, the frequency of specific binding cells is in vast excess of the frequency of antibody-forming cell precursors assessed by limiting dilution analysis [6]. The infinite variety of available antigens, and the high frequency of antigen-binding cells for any single antigen, suggest that antigen-binding cells are multispecific.

Functional studies, however, are consistent with antibody-forming cell precursors expressing restricted specificity for antigen. Using antigen binding as a functional marker for precursor cells, depletion [1–3] or enrichment [5, 7] of specific antigen-binding cells parallels the loss or gain of specific precursor activity respectively. However, such data are compatible with precursor cells being either monospecific or specific for a small number of distinct antigenic determinants. Although it is clear that not all antigen-binding cells are precursors, it is usually assumed that the functional specificity of the contained precursors is reflected in the specificity of antigen binding.

Raff et al. [8] demonstrated that when antigen receptors for polymerized flagellin (POL) are capped to one pole of a POL-binding cell, more than 90 % of the surface immunoglobulin (Ig) is co-capped. This suggests that such binding cells are monospecific since virtually all of the surface Ig, believed to be functioning as the antigen receptor, is involved in binding POL. The specificity of these POL-binding cells and their functional relevance as precursors for POL is now somewhat questionable, since POL has now been shown to have mitogenic properties [9, 10]. Since POL can stimulate precursors of unrelated specificity to differentiate into antibody production, it is not clear whether the membrane interactions reported by Raff can be compared to specific antigen interaction with membrane Ig.

A major problem in studies where the properties of antigen-binding cells are examined is the low frequency of such cells in unimmunized animals. In this report, which addresses the question of antigen-binding cell specificity, this problem was circumvented by employing an FACS to greatly enrich populations for antigen-binding cells.

Unprimed splenic lymphocytes were mixed with two complex antigens simultaneously, fluorescein-conjugated 2,4-dinitrophenyl-mouse IgG (^{F}DNP-MGG) and rhodamine-conjugated keyhole limpet hemocyanin (^{R}KLH). If cells can bind more than one distinct antigenic determinant, and if the receptors for any given antigenic determinant are distributed randomly on all cells, then the frequency of ^{R}KLH-binding cells should be similar for cells whether or not they bind ^{F}DNP-MGG, and the same would be true of the converse.

In the experiments reported here ^{F}DNP-MGG-binding cells and ^{R}KLH-binding cells were isolated using the FACS and examined by fluorescence microscopy for the presence of bound antigen of the alternate specificity. No double binding cells were found. These data support the contention that binding of one antigen precludes the binding of a second unrelated antigen, *i.e.* that antigen-binding cells are monospecific.

2. Materials and methods

2.1. Animals, preparation of cell suspensions and medium

Female BALB/cN mice bred at Stanford were used at the age of 4–6 months. Single spleen cell suspensions were prepared and contaminating erythrocytes and dead cells removed

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Abbreviations: POL: Polymerized flagellin FACS: Fluorescence-activated cell sorter MGG: Mouse IgG MSA: Mouse serum albumin DNP: 2,4-Dinitrophenyl DNP_{23} MGG: 2,4-Dinitrophenylated mouse IgG with an average of 23 molecules of DNP/molecules of MGG $^{F}DNP_{23}$ MGG: Fluorescein-conjugated DNP_{23}MGG KLH: Keyhole limpet hemocyanin ^{R}KLH: Rhodamine-conjugated KLH FDNB: 1-Fluoro-2,4-dinitrobenzene

as previously described [5]. Sodium azide (0.2 %) was included in media throughout staining and cell separation procedures.

2.2. Preparation of MGG, $^{F}DNP_{23}$MGG, DNP_{30} albumin and ^{R}KLH

Preparation of mouse IgG (MGG) and $^{F}DNP_{23}$MGG were as previously described [5]. Mouse albumin (MSA) was purified from normal BALB/cN serum by ion-exchange chromatography and conjugated with 1-fluoro-2,4-dinitrobenzene (FDNB) using the method described for MGG [5]. The resulting 2,4-dinitrophenylated albumin (DNP-MSA) had an average of 30 DNP groups/molecule of MSA*.

Keyhole limpet hemocyanin (KLH) obtained from Pacific Biomarine (Venice, California), was conjugated with tetramethylrhodamine and fractionated as previously described [11]. Rhodamine-conjugated KLH (^{R}KLH), with an average of 6 molecules of rhodamine/10^5 daltons of KLH, was used throughout these experiments.

2.3. Staining $^{F}DNP_{23}$MGG- and ^{R}KLH-binding cells

Staining concentrations of 0.167 mg/ml and 0.5 mg/ml of $^{F}DNP_{23}$MGG and ^{R}KLH, respectively, were used throughout with 2×10^7 cells in a final volume of 0.1 ml. Cells were washed through fetal calf serum (FCS) and then fresh medium after staining as previously described [5]. The same final concentration of both cells and labeled antigens was used when cells were exposed to both antigens simultaneously. For staining inhibition studies, to determine the specificity of $^{F}DNP_{23}$MGG-binding cells, all inhibitor concentrations were based on the molarity of DNP groups added. Inhibitors were added simultaneously with antigens.

2.4. Isolation of $^{F}DNP_{23}$MGG- and ^{R}KLH-binding cells by the FACS

The FACS used here (FACS-1, Becton-Dickenson Electronics, Mountain View, California) allows separation of cells according to fluorescence and light-scattering characteristics. Details of separation protocol and efficiency have been previously described [7].

The FACS can separate greater than 90 % pure populations of fluorescein-stained cells from an equal mixture of fluorescein- and rhodamine-stained cells of comparable fluorescent intensity sorting at up to 5000 cells/sec. However, when the rhodamine-stained cells were separated, the purity was only 50 %, with an equal contamination of fluorescein-stained cells.** Thus, using rhodamine excitation conditions with the laser line and photomultiplier tube currently available, the FACS does not distinguish between cells with equal intensities of rhodamine and fluorescein fluorescence. The presence of both fluorescein and rhodamine on a cell should not alter the sorting efficiency of the FACS if the amount of one of the bound conjugates is not decreased by the presence of the other.

* MSA concentration was determined using the Lowry procedure and the DNP concentration calculated based on the molar absorptivity $\epsilon = 1.74 \times 10^4$ at 360 nm.

** M.H. Julius, unpublished observation.

2.5. Fluorescence microscopy

Isolated binding cells were collected directly into 8 mm diameter, 8 mm-high glass cylinders sealed onto microscope slides and pelleted using a cytocentrifuge. Slides were fixed in 95 % ethanol for at least 20 min, air-dried and mounted in 9:1 glycerol:phosphate buffered saline, pH 8.5. The light source and filter combinations used for observing fluorescein and rhodamine fluorescence are as previously described [12, 13].

3. Results

3.1. DNP specificity of $^{F}DNP_{23}$MGG-binding cells in normal mouse spleen

Virtually all of the 1.4 % $^{F}DNP_{23}$MGG-binding cells stained using 0.167 mg/ml $^{F}DNP_{23}$MGG appear to be specific for DNP (Table 1). Inhibition of this large proportion of binding cells is virtually complete when large molar excesses of multivalent forms of DNP are present in the staining mixture. While the presence of a 100-fold molar excess of ε-DNP-lysine results in only a 29 % inhibition of staining, a 10-fold and 50-fold molar excess of DNP_{30}MSA based on DNP molarity, results in 84 % and 93 % inhibition respectively. A 5-fold molar excess of DNP groups as DNP_{23}MGG effects a 93 % inhibition of $^{F}DNP_{23}$MGG staining.

The greater efficiency of multivalent inhibitors indicates the very low affinity of the DNP receptors on many of those binding cells stained at this high concentration of $^{F}DNP_{23}$MGG.

The ability of MGG to inhibit 50 % of the $^{F}DNP_{23}$MGG-binding cells reflects a certain degree of nonspecificity of binding (Table 1). Lymphocytes might bind $^{F}DNP_{23}$MGG not only by virtue of DNP-specific receptors but via Fc receptors [14]

Table 1. DNP specificity of ^{F}DNP-MGG-binding cells

$^{F}DNP_{23}$MGG-labeled lymphocytes[a]	Inhibitor[b]	Molar excess of DNP on inhibitor[c]	Inhibition of binding of $^{F}DNP_{23}$MGG	^{R}KLH-labeled lymphocytes
(%)			(%)	(%)
1.4	–	–	–	0.4
1	ε-DNP-lysine	100	29	0.5
0.7	MGG[d]	–	50	0.4
0.4	ε-DNP-lysine	100	71	0.5
	MGG	–		
0.1	DNP_{23}MGG	5	93	0.5
0.1	DNP_{30}MSA	50	93	0.4
0.2	DNP_{30}MSA	10	84	0.4

a) Normal BALB/cN spleen cells were prepared and double stained with 0.167 mg/ml $^{F}DNP_{23}$MGG and 0.5 mg/ml ^{R}KLH as described in Section 2.

b) The staining procedure was modified by incorporating a molar excess of various forms of unlabeled DNP.

c) The molar excess of DNP in the form of inhibitor was calculated relative to the effective molarity of DNP in the form of $^{F}DNP_{23}$-MGG. Since there are 23 mol of DNP/mol (150 000 daltons) of MGG, 0.167 mg/ml $^{F}DNP_{23}$MGG represents 2.6×10^{-5} M DNP.

d) MGG was purified from BALB/cN normal serum by ion-exchange chromatography. It was tested as an inhibitor of $^{F}DNP_{23}$MGG binding by incorporating it at 10 mg/ml in the staining mixture.

which could react with available Fc determinants on the MGG moiety of the molecule. Thus, staining in the presence of excess MGG will saturate the Fc receptors and limit staining with $^{F}DNP_{23}$MGG to those cells with a sufficient density and affinity of DNP-specific receptors.

However, all the $^{F}DNP_{23}$MGG-binding cells detected are DNP-specific. Virtually complete inhibition of binding is obtained with excess DNP_{30}MSA (Table 1) in the absence of MGG. This suggests that many cells are detected as a result of co-operative binding of $^{F}DNP_{23}$MGG by two distinct receptors. Inhibition of binding by blocking either DNP-specific receptors or Fc receptors reduces the fluorescence of these cells below the (visual) detection threshold.

It follows that binding cells not inhibited by excess MGG must bind sufficient $^{F}DNP_{23}$MGG through DNP-specific receptors to exceed the visual fluorescence threshold.

Inhibition of $^{F}DNP_{23}$MGG-binding cells is specific (Table 1). Cells were simultaneously exposed to $^{F}DNP_{23}$MGG and ^{R}KLH in the presence or absence of inhibitors. The proportion of ^{R}KLH-binding cells observed ranged from 0.4–0.5 % and was unaffected by any of the concentrations of DNP ligands.

Simultaneous exposure of normal spleen cells to $^{F}DNP_{23}$MGG and ^{R}KLH does not significantly alter the frequency of the respective antigen-binding cells observed when the stains are used separately (Table 2). 1.2 % and 0.4 % of splenic lymphocytes bound detectable $^{F}DNP_{23}$MGG and ^{R}KLH respectively when aliquots of the same population of lymphocytes were stained. Simultaneous staining of the same aliquot resulted in 1.1 % and 0.5 % $^{F}DNP_{23}$MGG and ^{R}KLH-binding cells respectively.

3.2. A lack of $^{F}DNP_{23}$MGG and ^{R}KLH double binding cells in normal spleen

Isolation of $^{F}DNP_{23}$MGG-binding cells from a spleen cell suspension, which had been exposed to $^{F}DNP_{23}$MGG and ^{R}KLH simultaneously, resulted in a population with 92 % purity of $^{F}DNP_{23}$MGG-binding cells and a 1.9 % contamination of ^{R}KLH-binding cells as detected by fluorescence microscopy (Table 2). These purified cells were examined under rhodamine excitation conditions and each ^{R}KLH-binding cell was then examined for bound $^{F}DNP_{23}$MGG under fluorescein excitation conditions. None of the ^{R}KLH-binding cells had bound detectable $^{F}DNP_{23}$MGG. Of the 10 329 total cells counted (92 % of which should have been $^{F}DNP_{23}$MGG-binding cells) 287 were contaminating ^{R}KLH-binding cells on which no green fluorescence was seen (Table 2).

Assuming a random distribution of ^{R}KLH-specific receptors on lymphocytes, the purified $^{F}DNP_{23}$MGG-binding cell population was expected to contain 48 (0.5 % x 92 % x 10 329) $^{F}DNP_{23}$MGG-^{R}KLH double binding cells. This demonstrates the exclusion of receptors for ^{R}KLH on cells with receptors for $^{F}DNP_{23}$MGG.

Similarly, purified ^{R}KLH-binding cells do not bind detectable $^{F}DNP_{23}$MGG (Table 2). When ^{R}KLH-binding cells were isolated from the spleen cell suspension which had been simultaneously exposed to both antigens, only a 36 % purity of ^{R}KLH-binding cells (assessed by microscopy) was obtained. Rhodamine separation by the FACS with the filters used is not as specific as that for fluorescein (see above). All fluorescent cells are detected and sorted under these conditions, so the approximate 3:1 ratio of $^{F}DNP_{23}$MGG: ^{R}KLH-binding cells in the starting population explains the 55 % contamination with $^{F}DNP_{23}$MGG-binding cells.

Table 2. Isolation of $^{F}DNP_{23}$MGG and ^{R}KLH-binding cells: evidence for lack of DNP and KLH double binding cells

Label[a]	Number of cells counted[b]					Labeled cells		
	Total	F	R	F–R exp.	F–R obs.[c]	$^{F}DNP_{23}$MGG (%)	^{R}KLH (%)	$^{F}DNP_{23}$MGG + ^{R}KLH (%)
$^{F}DNP_{23}$MGG	1050	13	–	–	–	1.2	–	–
^{R}KLH	990	–	4	–	–	–	0.4	–
$^{F}DNP_{23}$MGG and ^{R}KLH	1000	11	5	nd[d]	nd	1.1	0.5	nd
$^{F}DNP_{23}$MGG and ^{R}KLH[e]	311	287	6	nd	nd	92	1.9	nd
	10329	nd	287	48	0[f]	nd	2.8	<0.01
$^{F}DNP_{23}$MGG and ^{R}KLH[g]	300	166	107	nd	nd	55	36	nd
	3127	nd	3127	34	0[f]	nd	nd	<0.03

a) Normal BALB/cN spleen cells were stained with $^{F}DNP_{23}$MGG at 0.167 mg/ml and/or ^{R}KLH at 0.5 mg/ml as described in Section 2.

b) Cells were first observed under white light darkfield illumination. Those with intact plasma membranes were observed under fluorescent illuminating conditions for both fluorescein (F) and rhodamine (R) as described in Section 2.

c) The expected number of cells binding both ^{R}KLH and $^{F}DNP_{23}$MGG in the purified $^{F}DNP_{23}$MGG-binding cell population was calculated assuming the frequency of ^{R}KLH-binding cells in the unfractionated population. Similarly the frequency of $^{F}DNP_{23}$MGG and ^{R}KLH double binding cells in the purified ^{R}KLH-binding cell population was calculated using the frequency of $^{F}DNP_{23}$MGG-binding cells in the unfractionated population.

d) Not done.

e) $^{F}DNP_{23}$MGG-binding cells were isolated and the purity determined using fluorescein excitation conditions. Cells were then counted under white light and rhodamine excitation conditions, and those cells binding ^{R}KLH were examined for the presence of $^{F}DNP_{23}$MGG.

f) 48 and 0; 34 and 0 are significantly different within the 95 % confidence interval.

g) ^{R}KLH-binding cells were isolated and the purity determined using first rhodamine excitation conditions followed by fluorescein excitation conditions. Since the contamination with $^{F}DNP_{23}$MGG-binding cells was high, ^{R}KLH-binding cells were counted directly and then examined for the presence of $^{F}DNP_{23}$MGG.

Again assuming a random distribution of $^{F}DNP_{23}MGG$ specific receptors on lymphocytes, 1.1 % of the 3127 ^{R}KLH-binding cells were expected to bind $^{F}DNP_{23}MGG$. No double binding cells were found: thus, there is an exclusion of $^{F}DNP_{23}MGG$ binding on cells binding ^{R}KLH.

4. Discussion

The objective of these experiments was to test whether antigen-binding lymphocytes in the spleens of unimmunized animals are monospecific. To do so, splenic lymphocytes were first simultaneously exposed to two contrastingly fluorescent unrelated antigens. Cells binding one of the antigens were then purified using the FACS and subsequently analyzed for the presence of the second antigen. Using this procedure, it was possible to examine 13 000 cells binding a given antigen for the binding of a second antigen. The absence of double binding cells strongly suggests that antigen-binding cells are monospecific.

Cells binding multiple antigens can be explained in a variety of ways. Such cells could have a single type of cross-reactive receptor molecule capable of reacting with a wide range of antigen specificities. Alternatively, several different types of receptor molecules, each with a unique specificity, might be expressed on each cell. Perhaps a mechanism intermediate between the above alternatives might exist. Since double binders were not found, none of these possibilities exists for the antigens DNP and KLH.

The techniques employed in this study do not allow one to distinguish a cell binding very little antigen from one binding no antigen. Thus, double binding cells would not be detected if they bound vastly unequal amounts of the two antigens tested, due to either one antigen displacing the other or perhaps very low avidity receptors for one of the antigens.

Functional studies suggest that DNP-binding cells isolated from unprimed mice and KLH-binding cells isolated from KLH-primed mice using the same staining concentrations reported here, contain all of the precursor cells present in the respective unfractionated spleen [5, 12]. It is, therefore, unlikely that we have missed detecting a functional population of low avidity binding lymphocytes which contains the potential double binders.

The frequency of KLH-binding cells detected in unprimed spleens is the same as in primed spleens, using the staining concentration reported here. However, we have not directly demonstrated that all the KLH precursors in unprimed spleens are stained at this concentration of antigen.

In the preliminary stages of these studies it was noted that a preparation of ^{R}KLH, not subsequently used, labeled about 0.5 % of spleen cells with a normal morphology, size and pattern of staining. However, such cells could not be isolated using the FACS under standard separation conditions, in which dead cells, as detected by light scattering [7], are excluded. When separation conditions were altered to include dead cells, nearly pure populations of these aberrant ^{R}KLH-binding cells were obtained. It has not been determined whether the ^{R}KLH was specifically killing binding cells, or labeling cells that were already dead. Since these cells looked identical to viable antigen-binding cells by the criteria of cell morphology, size and staining pattern, these criteria for viability may be inadequate.

The high frequency of cells binding a single antigen, and the absence of double staining cells, appear to be paradoxical in that not enough monospecific cells would be available to respond to all possible antigens. These results are probably best explained by the great variety of distinct antigenic determinants present on the antigens employed. These antigens are therefore, labeling cells of a great many unique specificities. Also, if a cell bears a single type of receptor which can bind both antigens, but with a large difference in affinity for one of the antigens, it would not be detected as a double binding cell using the staining technique we employed. Since both antigens are present simultaneously during the staining incubation, the competition for receptors results in a selection for the antigen which binds most avidly, displacing those antigens less avidly bound.

In conclusion, these results support the contention that antigen-binding cells and hence precursors of antibody-forming cells in nonimmune mice are monospecific. The data are consistent with a variety of functional studies demonstrating restricted antigen specificity of precursor cells. Thus, the depletion [1–3] or enrichment [5, 7] of antigen-binding cells of one antigen specificity results in the depletion or enrichment of precursor activity for that antigen specificity only. If multispecific antigen-binding cells exist, and reflect the multispecificity of precursor cells, it is unlikely that removal of binding cells with one antigen specificity would result in the loss of precursor activity for only that specificity. The presence of monospecific receptors on antibody-forming precursor cells is further supported indirectly by studies demonstrating allelic exclusion of Ig allotypes on rabbit lymphocytes [15, 16] and the presence of a single idiotype associated with different isotypes simultaneously on the surface of human lymphoma cells [17–19].

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