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 contrasting 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.

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 FcR binding cells 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 producing, 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 FCS 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 (FDPN-MGG) and rhodamine-conjugated keyhole limpet hemocyanin (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 KLH-binding cells should be similar for cells whether or not they bind FDPN-MGG, and the same would be true of the converse.

In the experiments reported here FDPN-MGG-binding cells and 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/c or C3H 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.
as previously described [5]. Sodium azide (0.2 %) was included in media throughout staining and cell separation procedures.

2.2. Preparation of MGG, DNP32 MGG, DNP30 albumin and RKLH

Preparation of mouse IgG (MGG) and DNP32 MGG were as previously described [5]. Mouse albumin (MSA) was purified from normal BALB/c mouse 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 (RKLH), with an average of 6 molecules of rhodamine/10^3 daltons of KLH, was used throughout these experiments.

2.3. Staining DNP32 MGG- and RKLH-binding cells

Staining concentrations of 0.167 mg/ml and 0.5 mg/ml of DNP32 MGG and 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 DNP32 MGG-binding cells, all inhibitor concentrations were based on the morality of DNP groups added. Inhibitors were added simultaneously with antigens.

2.4. Isolation of DNP32 MGG- and RKLH binding cells by the FACS

The FACs used here (FACS-1, Becton-Dickinson 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 dyes, and conjugates is not decreased by the presence of the other.

- [5] sodium azide

2.5. Fluorescence microscopy

Isolated binding cells were collected directly into 8 mm diameter, 5 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 DNP32 MGG-binding cells in normal mouse spleen

Virtually all of the 1.4 % DNP32 MGG-binding cells stained using 0.167 mg/ml DNP32 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 e-DNP-lysine results in only a 29 % inhibition of staining, a 10-fold and 50-fold molar excess of DNP30 MSA based on DNP molarity, results in 84 % and 93 % inhibition respectively. A 5-fold molar excess of DNP groups as DNP32 MGG effects a 93 % inhibition of DNP32 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 DNP32 MGG.

The ability of MGG to inhibit 50 % of the DNP32 MGG-binding cells reflects a certain degree of nonspecificity of binding (Table 1). Lymphocytes might bind DNP32 MGG not only by virtue of DNP-specific receptors but via Fe receptors [14] by virtue of DNP-specific receptors but via Fe receptors [14].

Table 1. DNP specificity of DNP32 MGG-binding cells

<table>
<thead>
<tr>
<th>DNP32 MGG-labeled lymphocytes</th>
<th>Inhibitor(a)</th>
<th>Molar excess of DNP on inhibitor(b)</th>
<th>Inhibition of binding of DNP32 MGG</th>
<th>RKLH-labeled lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>0.1</td>
<td>DNP32 MGG</td>
<td>5</td>
<td>93</td>
<td>0.5</td>
</tr>
<tr>
<td>0.1</td>
<td>DNP30 MSA</td>
<td>50</td>
<td>93</td>
<td>0.4</td>
</tr>
<tr>
<td>0.2</td>
<td>DNP30 MSA</td>
<td>10</td>
<td>84</td>
<td>0.4</td>
</tr>
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</table>

a) Normal BALB/c mouse spleen cells were prepared and double stained with 0.167 mg/ml DNP32 MGG and 0.5 mg/ml RKLH 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 DNP32 MGG. Since there are 23 mol of DNP/mol (150 000 daltons) of MGG, 0.167 mg/ml DNP32 MGG represents 2.6 × 10^-5 M DNP.

MGG was purified from BALB/c normal serum by ion-exchange chromatography. It was tested as an inhibitor of DNP32 MGG binding by incorporating it at 10 mg/ml in the staining mixture.

- [7] [11] [12] [13] [14]

- [14] [14]
which could react with available Fe determinants on the MGG moiety of the molecule. Thus, staining in the presence of excess MGG will saturate the Fe receptors and limit staining with FDP3, MGG to those cells with a sufficient density and affinity of DNP-specific receptors.

However, all the FDP3, MGG-binding cells detected are DNP-specific. Virtually complete inhibition of binding is obtained with excess DNP, MSA (Table 1) in the absence of MGG. This suggests that many cells are detected as a result of cooperative binding of FDP3, MGG by two distinct receptors. Inhibition of binding by blocking either DNP-specific receptors or Fe 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 FDP3, MGG through DNP-specific receptors to exceed the visual fluorescence threshold.

Inhibition of FDP3, MGG-binding cells is specific (Table 1). Cells were simultaneously exposed to FDP3, MGG and RKLH in the presence or absence of inhibitors. The proportion of RKLH-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 FDP3, MGG and RKLH 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 FDP3, MGG and RKLH respectively when aliquots of the same population of lymphocytes were stained. Simultaneous staining of the same aliquot resulted in 1.1 % and 0.5 % FDP3, MGG and RKLH-binding cells respectively.

Table 2. Isolation of FDP3, MGG and RKLH-binding cells: evidence for lack of DNP and RKLH double binding cells

<table>
<thead>
<tr>
<th>Label(s)</th>
<th>Total</th>
<th>F</th>
<th>R</th>
<th>F–R exp. ob.</th>
<th>FDP3, MGG</th>
<th>RKLH</th>
<th>FDP3, MGG + RKLH</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDP3, MGG</td>
<td>1050</td>
<td>13</td>
<td>-</td>
<td>-</td>
<td>1.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RKLH</td>
<td>990</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FDP3, MGG and RKLH</td>
<td>1000</td>
<td>11</td>
<td>5</td>
<td>nd</td>
<td>1.1</td>
<td>0.5</td>
<td>nd</td>
</tr>
<tr>
<td>FDP3, MGG and RKLH</td>
<td>311</td>
<td>287</td>
<td>6</td>
<td>nd</td>
<td>92</td>
<td>1.9</td>
<td>nd</td>
</tr>
<tr>
<td>FDP3, MGG and RKLH</td>
<td>16329</td>
<td>nd</td>
<td>287</td>
<td>48</td>
<td>2.8</td>
<td>&lt;0.01</td>
<td>-</td>
</tr>
<tr>
<td>RKLH</td>
<td>300</td>
<td>166</td>
<td>107</td>
<td>nd</td>
<td>55</td>
<td>36</td>
<td>nd</td>
</tr>
<tr>
<td>RKLH</td>
<td>3127</td>
<td>nd</td>
<td>3127</td>
<td>34</td>
<td>0.01</td>
<td>-</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

a) Normal BALB/c spleen cells were stained with FDP3, MGG at 0.167 mg/ml and/or RKLH 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 fluorescein illuminating conditions for both fluorescein (F) and rhodamine (R) as described in Section 2.

c) The expected number of cells binding both RKLH and FDP3, MGG in the purified FDP3, MGG-binding cell population was calculated assuming the frequency of RKLH-binding cells in the unfractionated population. Similarly the frequency of FDP3, MGG and RKLH double binding cells in the purified RKLH-binding cell population was calculated using the frequency of FDP3, MGG-binding cells in the unfractionated population.

d) Not done.

e) FDP3, 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 RKLH were examined for the presence of FDP3, MGG.

f) 48 and 0.34 and 0.04 are significantly different within the 95 % confidence interval.
g) RKLH-binding cells were isolated and the purity determined using white rhodamine excitation conditions followed by fluorescein excitation conditions. Since the contamination with FDP3, MGG-binding cells was high, RKLH-binding cells were counted directly and then examined for the presence of FDP3, MGG.
4. Discussion

The objective of these experiments was to test whether antigen-binding lymphocytes in the spleens of immunized 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 vestigial 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 unfunctioned spleen [5, 12]. If this is true, it is 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 KLH, not subsequently used, labeled about 15% 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 KLH-binding cells were obtained. It has not been determined whether the 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 allelotypes on rabbit lymphocytes [15, 16] and the presence of a single idotype associated with different isotypes simultaneously on the surface of human lymphoma cells [17-19].

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5. References