ISOLATION OF ANTIGEN-BINDING CELLS FROM UNPRIMED MICE

Demonstration of Antibody-Forming Cell Precursor Activity and Correlation between Precursor and Secreted Antibody Avidities*

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The time-dependent increase in the average affinity of serum antibody after immunization (1), is said to be a result of antigen-driven selection of those antibody-forming cell precursors having the highest affinity antigen receptors (2). This interpretation infers that the specificity and affinity of antigen-binding receptors on antibody-forming cell precursors are directly correlated to the serum antibody produced by progeny antibody-secreting cells.

It has been found that changes in the affinity of serum antibody with time after immunization reflect changes in the population of antibody-secreting cells. By determining the concentration of antigen required to inhibit individual plaque-forming cells (PFC)[1] assayed at various times after immunization, a time-dependent avidity increase at the antibody-secreting cell level is seen and is comparable to the increase in affinity found in serum antibody (3). Thus, a good correlation between the (intrinsic) affinity of serum antibody and the avidity of the antibody-secreting cell products has been established.

In contradistinction, evidence correlating the avidity of antigen receptors on antibody-secreting cell precursors, with the avidity of antibody secreted by their progeny cells, is meager. Indirect evidence has been reported demonstrating that memory cells with high avidity antigen-binding receptors are required to give rise to high avidity antibody-secreting cells (4). However, the putatively high avidity precursors were not isolated and tested directly in these experiments.

To directly determine the relationship between the avidity of secreted antibody and the avidity of antigen receptors on precursors of antibody-secreting cells requires the isolation of precursor cells with different antigen avidities and measurement of the avidities of antibody-secreting cells derived from these

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[1] *Abbreviations used in this paper:* DNP-MGG, 2,4-dinitrophenyl mouse gamma globulin; ^{F}DNP-MGG, fluorescein-conjugated DNP-MGG; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; F/P, fluorescein per protein; KLH, keyhole limpet hemocyanin; ^{R}KLH, rhodamine-conjugated KLH; PBS, phosphate-buffered saline; PFC, plaque-forming cell(s); TNP, 2,4,6-trinitrophenyl.

precursors on antigen stimulation. We have already directly demonstrated that populations of antigen-binding cells from immune (5) and nonimmune (6) animals contain the precursors of antibody-secreting cells. Others have found direct evidence that antigen-binding cells isolated from both nonimmune mice on affinity columns (7) and from immune mice on antigen-derivatized nylon fibers (8) contain antigen-specific precursors required to give adoptive primary and secondary immune responses, respectively. To date, neither of the latter methods have provided adequate purity of precursor activity or enabled purification of precursors with a known range of antigen-binding avidities, both of which are required for a precursor-product correlation study.

The development of a fluorescence-activated cell sorter (FACS) (9) has enabled the isolation of virtually pure populations of viable antigen-binding cells with full functional activity (5, 6). We have isolated cells with low, medium, and high binding avidity for DNP by staining normal spleen cells with fluorescein-ated DNP-mouse gamma globulin (^{F}DNP-MGG) and separation using a FACS.

It has been postulated that precursors of low affinity antibody-secreting cells require higher concentrations of antigen to bind and be stimulated than do precursors of high affinity antibody-secreting cells (1). Therefore, high avidity cells were stained using low concentrations of ^{F}DNP-MGG while low and medium avidity cells were stained using moderately high concentrations in the presence of various concentrations of the univalent competing ligand ϵ-DNP-lysine (10, 11).

The precursor activity of the purified DNP-binding cells was tested by transferring these cells together with DNP-keyhole limpet hemocyanin (KLH) and a source of KLH carrier-primed cooperator cells into irradiated recipients and subsequently assaying the recipient spleens for anti-DNP-plaque-forming cells (PFC). In addition to quantitating the precursor activity of the purified DNP-binding cells, the hemolytic plaque assay was used to study the avidity of single antibody-secreting cells (3). By determining the avidity of anti-DNP-PFC resulting from the transfer of purified DNP-binding cells with varying avidities for ^{F}DNP-MGG, we have found a direct correlation between the avidity of the DNP antigen-binding cells and the anti-DNP antibody secreted by the PFC derived from them.

Materials and Methods

Animals. Male and female mice of the congenic strains BALB/cN and BAB/14 Hz were used at the age of 2–6 mo (BAB/13 were kindly supplied by Dr. M. Potter (NCI, NIH) and BAB/14 were derived in this laboratory by one further back-cross to BALB/cN and subsequent repeated inbreeding of the resulting heterozygotes).

Preparation of Cell Suspensions and Media. Single cell suspensions of spleen were prepared as previously described (5). Cell suspensions in preparation for loading onto nylon wool columns, cell separation, and immunofluorescent staining were prepared in Dulbecco's phosphate-buffered saline (PBS), pH 7.5, (12) supplemented with 5% heat-inactivated fetal calf serum (FCS). Before processing spleen cell suspensions through the FACS, erythrocytes were lysed by incubating the cells for 5 min at 0°C in Gey's balanced salt solution (13) in which the NaCl was replaced with an equimolar concentration of NH_4Cl. Spleen cells in preparation for plaquing were prepared in MEM with Hanks' balanced salt solution (14). FCS wherever used was first inactivated at 56°C for 30 min.

Preparation of Carrier-Primed Splenic T Cells, Indirect Immunofluorescence Staining for T and Ig-Bearing Cells and Fluorescence Microscopy. As previously described (5, 15).

Preparation of ^{F}DNP-MGG and Staining of DNP-Binding Cells. MGG was purified from BALB/cN normal serum by ion-exchange chromatography and conjugated with 1-fluoro-2-dinitrobenzene (Sigma Chemical Co., St. Louis, Mo.) at pH 8.0 in 0.5 M $NaHCO_3$ for 2–3 h at room temperature. The resulting DNP-MGG contained an average of 23 DNP groups/molecule of MGG.[2] Fluorescein conjugates of DNP-MGG were prepared using fluorescein isothiocyanate (16). The ^{F}DNP-MGG was fractionated by a gradient elution from DEAE-cellulose (17, 18). Fractions with fluorescein per protein (F/P) ratios between 2 and 5 were pooled and concentrated by ultrafiltration. ^{F}DNP-MGG with an F/P ratio of 3.5 was used throughout these experiments.

Staining concentrations of ^{F}DNP-MGG were varied from 6 to 1,600 μg/ml, 0.1 ml of the appropriate ^{F}DNP-MGG concentration was added/2 × 10^7 pelleted cells. Subsequent to a 20 min incubation at 22–25°C the cells were pelleted through neat FCS and then washed once in an excess of medium.

A modification of the above staining procedure was used to determine the relative avidity of ^{F}DNP-MGG-binding cells. A 10- to 100-fold molar excess of ε-DNP-lysine (relative to the molarity of DNP present on the ^{F}DNP-MGG) was included in the staining mixture. The proportion of binding cells observed by fluorescent microscopy in the presence and absence of ε-DNP-lysine was determined and the percent inhibition of staining calculated.

To demonstrate specificity of inhibition, cells were simultaneously stained with ^{F}DNP-MGG in the presence of various hapten concentrations and with an unrelated antigen, contrastingly fluorescent rhodamine-conjugated KLH (^{R}KLH) (using 0.1 ml of ^{R}KLH at 0.5 mg/ml/2 × 10^7 cells). The proportion of ^{R}KLH-binding cells observed was unaffected by any of the ε-DNP-lysine concentrations used.

Purification of ^{F}DNP-MGG-Binding Cells by the FACS. The FACS used here allows separation of cells according to fluorescence, light-scattering characteristics, or selected combinations of these two parameters. Details of separation protocol and efficiency have been previously described (5, 6). A typical separation yields two fractions of cells, an enriched fraction containing greater than 85% ^{F}DNP-MGG-binding cells and a depleted fraction containing 500-fold fewer ^{F}DNP-MGG-binding cells compared to the unseparated spleen.

Antigens. DNP-KLH was prepared by reacting 1-fluoro-2,4-dinitrobenzene (Sigma Chemical Co.) at pH 8.0, in 0.5 M $NaHCO_3$ with KLH (Pacific Bio-Marine Supply Co., Venice, Calif.) for 2–3 h at room temperature. Molar ratios of DNP/10^5 daltons of KLH were calculated[3] and $DNP_{8\text{-}10}$-KLH was used in these experiments. Aliquots of DNP-KLH (prepared as described above) were alum precipitated at pH 6.5 with a 9% solution of $ALK(SO_4)_2$ for 3 h at room temperature.

Irradiation and Adoptive Transfers. Recipients in adoptive transfer experiments received 600 rads whole-body X irradiation, 16–18 h before receiving cells. A modification of the Mitchison hapten-carrier transfer protocol (19, 20) was used. Various combinations of normal spleen cells, purified ^{F}DNP-MGG-binding cells, spleen cells depleted of ^{F}DNP-MGG-binding cells and KLH-primed splenic T cells were injected intravenously. 100 μg of alum-precipitated DNP-KLH was given intraperitoneally immediately after injection of cells and 10 μg of aqueous DNP-KLH was given intravenously on day 5. Recipients were bled and sacrificed on day 12, serum and cell suspensions of recipient spleens were assayed for anti-KLH antibody and DNP-PFC, respectively.

DNP-PFC Assay and Inhibition with ε-DNP-Lysine for Determination of Relative Hapten Avidities. Anti-DNP antibody-secreting cells (PFC) were measured using a modified version of the hemolytic plaque technique (21). SRBC were conjugated with 2,4,6-trinitrobenzene-sulphonic acid (Eastman Organic Chemicals Div., Eastman Kodak Co., N. Y.) (22). Virtually no PFC were observed using unconjugated SRBC. Fresh frozen guinea pig serum at a final concentration of 1/24 was used as a complement source. In early experiments a polyvalent rabbit antimouse antiserum was used to facilitate hemolysis. Since substantial numbers of indirect PFC were not found, in subsequent experiments only direct PFC were measured. We have detected identical numbers of DNP-PFC in experiments where the same spleens were assayed using either TNP-SRBC (22) or DNP-SRBC (23) as indicators. Due to the ease of preparation, TNP-SRBC were used in subsequent experiments.

The plaque inhibition method of Andersson (3) was used to determine the relative avidity for DNP of the PFC. The term "avidity" rather than "affinity" is used here since it is not clear how accurately

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[2] Based on $E^{1\%}_{1\,cm}$ = 14 at 280 nm and $\epsilon_M = 1.74 \times 10^4$ at 360 nm for MGG and DNP, respectively.

[3] Using $E^{1\%}_{1\,cm}$ = 15.5 at 280 nm and $\epsilon_M = 1.74 \times 10^4$ at 360 nm for KLH and DNP, respectively.

an affinity constant (Ka) (24) can be determined in this way. Concentrations of ε-DNP-lysine ranging from 10^{-4} to 10^{-7} M were included in some plaquing chambers. High avidity antibody is saturated at lower concentrations of ε-DNP-lysine than low avidity antibody. Therefore, low concentrations of ε-DNP-lysine prevent high avidity antibody from lysing TNP-SRBC, whereas higher concentrations of ε-DNP-lysine would be required to block hemolysis by low avidity antibody. Strictly, a weighted product of antibody avidity and amount secreted per cell is measured by ε-DNP-lysine inhibition. However, since the sizes of uninhibited PFC varied less than about 50% in diameter, while the inhibitions vary over a 10^3 concentration range of inhibitor, the major parameter measured is relative avidity rather than amount per cell. In some experiments for assaying very low avidity DNP-PFC, multivalent DNP (DNP-MGG) was used as an inhibitor and was varied from 10^{-5} to 10^{-8} M DNP in the plaquing chambers. Inhibition of PFC with either ε-DNP-lysine or DNP-MGG was specific for DNP-PFC and did not reduce the number of SRBC-PFC detected on day 4 of a primary response to 4×10^8 SRBC.

Titrations. Anti-KLH serum titers were individually determined using the passive microhemagglutination technique (25). KLH was coupled to SRBC using a modification of the glutaraldehyde method (26). Sera were heat inactivated at 56°C for 30 min before titration.

Results

Adoptive Primary Response to DNP-KLH. The DNP precursor activity contained in unprimed spleen was determined using an adoptive syngeneic or congenic hapten-carrier transfer system (19). Graded numbers of unprimed spleen cells and KLH-primed, nylon column-purified (15) T cells were mixed and transferred into irradiated recipients with antigen (see Materials and Methods).

The DNP-PFC response of irradiated recipients reconstituted with normal histocompatible spleen cells is substantially improved by supplementation with carrier-primed cooperator cells (27). Fig. 1 *a* shows the DNP-PFC response obtained on transfer of graded numbers of normal spleen cells with or without addition of a constant number of KLH-primed splenic T cells. Addition of 7.5×10^6 KLH-primed and nylon-purified (15) cooperators results in a greater than 10-fold increased response at each dose of normal spleen tested. Thus in normal spleen, cooperators for the DNP-KLH response are limiting on transfer.

The increase in response of a fixed number of normal spleen cells transferred as a function of the number of carrier-primed T cells added is shown in Fig. 1 *b*. There is about a 10-fold greater than additive increase in response with addition of 5×10^6 KLH-primed splenic T cells. This increase must be due to the expression of DNP-PFC precursors derived from the normal spleen, since the response of 5×10^6 T cells transferred alone could have accounted for only 10% of the response of the mixture of normal spleen and T cells.

The addition of 5×10^6 cooperators allows maximal expression of the DNP-PFC precursors in 5×10^6 normal spleen cells. When 1×10^7 carrier-primed T cells are added (in another experiment), there is no further increase in the number of PFC observed (26×10^3 PFC/spleen with 5×10^6 T cells and 29×10^3 PFC/spleen with 1×10^7 T cells.) In experiments which follow, 7.5×10^6 carrier-primed splenic T cells were added to populations to be tested for precursor activity in order to assure an excess of cooperator activity.

^{F}DNP-MGG-Binding Cells in Normal Mouse Spleen. The proportion of ^{F}DNP-MGG-binding cells in normal spleen detectable by fluorescence microscopy increases with the staining concentration of ^{F}DNP-MGG (Table I). It reaches a plateau of about 2.5% at a staining concentration of ^{F}DNP-MGG between 500 and 1,600 μg/ml.

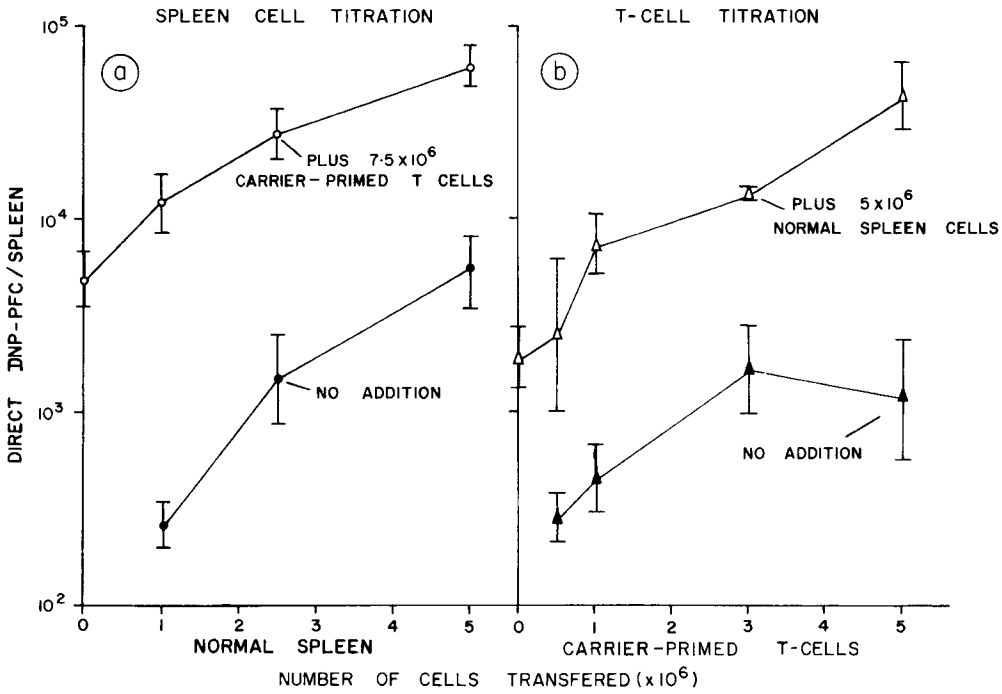

FIG. 1. (*a*) Adoptive primary dose response to DNP-KLH. Irradiated BAB/14 mice were injected with either normal BALB/cN spleen cells alone or in conjunction with 7.5×10^6 7-day KLH-primed nylon column-purified splenic T cells (obtained from BALB/cN spleen and contained 92% T cells and 4% Ig-bearing cells as assessed by immunofluorescence). Animals received 100 μg alum-precipitated DNP-KLH intraperitoneally on the day of transfer, boosted with 10 μg aqueous DNP-KLH intravenously on day 5, and sacrificed on day 12. Each point represents the geometric mean of the responses of four animals and one SE about the mean is indicated. (*b*) Dose response of carrier-primed T cells. Irradiated BAB/14 mice were injected with either 2.5-mo KLH-primed nylon column-purified splenic T cells (obtained from BALB/cN spleen and contained 89% T cells and 4% Ig-bearing cells as assessed by immunofluorescence) alone or in combination with 5×10^6 normal BALB/cN spleen cells. See legend Fig. 1 *a* for immunization regime. Each point represents the geometric mean of the response of four animals and one SE about the mean is indicated.

TABLE I

Effect of ^{F}DNP-MGG Concentration on the Number of Labeled Lymphocytes in Normal Mouse Spleen

Staining concentration* of ^{F}DNP-MGG	No. of lymphocytes‡ counted ($\times 10^2$)	Labeled lymphocytes
$\mu g/ml$		%
6	101	0.03
18	51	0.1
55	19	0.3
167	15	1.6
500	15	2.7
1,600	15	2.3

* Normal BALB/cN spleen cells were prepared and stained with ^{F}DNP-MGG as described in the Materials and Methods.

‡ Cell smears were first examined under white-light darkfield illumination and only those cells with intact plasma membranes exhibiting speckled, ringed, or capped membrane-associated fluorescence were considered positive.

The increased proportion of binding cells with the ^{F}DNP-MGG concentration reflects a wide range of avidity in the binding population as well as a certain degree of nonspecificity. High avidity binding cells (i.e., those which stain at low ^{F}DNP-MGG concentrations) are specific for DNP. A 100-fold molar excess of ϵ-DNP-lysine reduced the number of detectable binding cells to 15% of the control (see Table II). However, binding by low avidity cells showed less

TABLE II
Specific Inhibition of ^{F}DNP-MGG-Binding Cells

Staining concentration* ^{F}DNP-MGG	Molarity of DNP‡ on ^{F}DNP-MGG $\times 10^{-6}$ M	Molarity of ϵ-DNP-lysine§ in staining mixture $\times 10^{-5}$ M	^{F}DNP-MGG‖-labeled lymphocytes	Inhibition of binding of ^{F}DNP-MGG
μg/ml			%	%
167	27	—	1.0	—
		27	0.9	10
		270	0.8	20
55	9	—	0.38	—
		9	0.33	25
		90	0.20	50
18	3	—	0.20	—
		3	0.12	40
		30	0.08	60
6	1	—	0.07	—
		1	0.02	71
		10	0.01	86

* Normal BALB/cN spleen cells were prepared and double stained with ^{F}DNP-MGG and ^{R}KLH as described in the Materials and Methods. 500 μg/ml of ^{R}KLH was included in all staining mixtures. The percent of ^{R}KLH-labeled lymphocytes ranged from 0.20 to 0.28% in tubes not containing ϵ-DNP-lysine. None of the concentrations of ϵ-DNP-lysine used in inhibitions significantly decreased the number of ^{R}KLH-binding lymphocytes. The percent of ^{R}KLH-labeled lymphocytes observed in the presence of from 1 to 27 $\times 10^{-5}$ M ϵ-DNP-lysine ranged from 0.20 to 0.26%.

‡ The effective molarity of DNP present in varying concentrations of ^{F}DNP-MGG was calculated on the basis of 23 mol of DNP/mol (150,000 daltons) of MGG.

§ Either a 10- or 100-fold molar excess of ϵ-DNP-lysine (based on the molarity of DNP present in the form of ^{F}DNP-MGG) was added to the staining mixtures for inhibition studies.

‖ Rhodamine-labeled glutaraldehyde-fixed chicken erythrocytes (which fluoresce under both fluorescein and rhodamine illumination conditions) were added to the stained spleen cells in a ratio of 1:200 before smears were prepared. Slides were scanned for ^{F}DNP-MGG-binding cells and the proportion of labeled lymphocytes calculated based on the number of chicken erythrocytes counted. The same procedure was used to determine the proportion of ^{R}KLH-binding lymphocytes. Between 5,000 and 30,000 lymphocytes were scanned on each slide.

specificity in that a 100-fold molar excess of ϵ-DNP-lysine at the highest staining concentration reduced the number of detectable binding cells only to 80% of the control. Recent experiments[4] indicate that a major component of this nonspecificity is due to binding of the MGG moiety of the ^{F}DNP-MGG.

[4] Julius, M. H. Unpublished observations.

The Adoptive Primary Response of Isolated ^{F}DNP-MGG-Binding Cells. 2% of normal BALB/cN splenic lymphocytes bound detectable ^{F}DNP-MGG at a high-staining concentration (167 μg/ml). Isolation of these cells using the FACS yielded a population containing 91% ^{F}DNP-MGG-binding cells and a ninefold depleted population containing 0.3% binding cells (see Table III). These populations were assayed for DNP-PFC precursor activity in adoptive transfer experiments with carrier-primed T cells. Transfer without carrier-primed T cells resulted in low DNP-PFC responses (see Table III).

To test for enrichment of precursor activity, the number of isolated binding

TABLE III
Adoptive Primary Response of Purified ^{F}DNP-MGG-Binding Cells

% Fluorescent cells* in fractions			No. of cells transferred ($\times 10^6$)‡				Direct anti-DNP§ PFC/spleen ($\times 10^3$)	Total anti-KLH‖ titer ($\log_2$)
Unfractionated	^{F}DNP-MGG (+)	^{F}DNP-MGG (−)	KLH-primed T¶	Unfractionated	^{F}DNP-MGG (+)	^{F}DNP-MGG (−)		
2.0	91	0.3	—	5	—	—	6 (4–9)	2.0 ± 0
			7.5	—	—	—	6 (6–7)	3.4 ± 0.6
			—	—	0.1	—	1 (0.8–1)	3.5 ± 0.5
			—	—	—	5	1 (0.5–2)	4.0 ± 0
			7.5	5	—	—	59 (52–67)	8.5 ± 2.9
			7.5	—	0.1	—	27 (23–30)	4.0 ± 0
			7.5	—	—	5	28 (24–33)	8.5 ± 2.9

* BALB/cN normal spleen cells were stained with ^{F}DNP-MGG at 167 μg/ml. DNP-MGG-positive and -negative cells were isolated using the FACS as described in the Materials and Methods.

‡ Unfractionated, ^{F}DNP-MGG (+), or ^{F}DNP-MGG (−) cell populations were transferred into 600 R irradiated BAB/14 mice intravenously. Animals received 100 μg alum-precipitated DNP-KLH on day 0, 10 μg aqueous DNP-KLH on day 5, and were bled and sacrificed on day 12.

§ Geometric mean of the responses of four animals. Numbers in parentheses represent one SE about the mean.

‖ $\log_2$ anti-KLH hemagglutination titer ± SE. Each number represents the arithmetic mean of the titers of four animals.

¶ KLH-primed T cells were isolated from 7-day primed BAB/14 spleens by nylon wool passage and contained 93% T cells and 1.4% Ig-bearing cells as assessed by immunofluorescent staining.

cells transferred from the enriched fraction was adjusted to be roughly equivalent to the number of binding cells found in 5×10^6 unfractionated spleen. Thus, in this experiment 1×10^5 isolated binding cells (at 91% purity) were transferred since the unfractionated population contained 2% binding cells. As seen by comparing lines 5 and 6 of Table III, 10^5 cells of the DNP (+) fraction gave rise to slightly fewer DNP-PFC (actually half as many in this experiment) as 50 times more unfractionated cells. Thus, the precursor activity in the antigen-binding cells is greatly enriched.

To assess depletion of precursor activity, equal numbers of the unfractionated and the DNP-negative cells were transferred along with T cells. Comparison of the last two lines of Table III shows no apparent depletion. However (see below), there is a striking qualitative difference in the PFC obtained from the two populations.

The enrichment for DNP-PFC precursors was specific in that it did not give a concomitant enrichment for anti-KLH precursors. The DNP (+) fraction gave a KLH response not above the background from KLH-primed B cells contaminating the T cells. Furthermore, the depleted fraction contained the same anti-KLH precursor activity as unfractionated spleen (see Table III).

Relationship Between the Avidity of ^{F}DNP-MGG Binding DNP-PFC Precursor Cells and the DNP-PFC Resulting on Transfer of Purified Binding Cells. There is a striking qualitative difference in the DNP-PFC obtained from the depleted fraction and the DNP-PFC obtained from either the isolated binding cells or unfractionated spleen. The avidities of the DNP-PFC in the recipients of the depleted fraction are about 300-fold lower.

The avidities of DNP-PFC were determined by sensitivity to inhibition by either ε-DNP-lysine or DNP-MGG. The data in Fig. 2 *a* show that while only 45% of the DNP-PFC in recipients of cells from the depleted fraction are inhibited at the highest concentration of ε-DNP-lysine (10^{-4} M), the same concentration inhibited 90% of the DNP-PFC in recipients of unfractionated spleen. By interpolation, 45% of the DNP-PFC in recipients of unfractionated spleen were inhibited at about 3×10^{-7} M ε-DNP-lysine, a factor of 300 higher. All of the DNP-PFC in the recipients of cells from the depleted fraction are inhibited by DNP_{23}-MGG indicating that even the lowest avidity PFC are specific for DNP. (The multivalency of DNP on DNP-MGG makes it a more efficient inhibitor

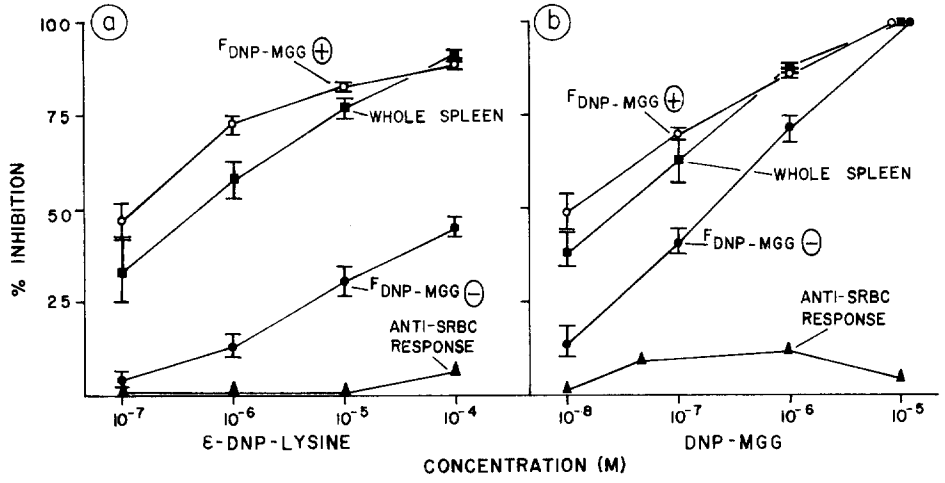

FIG. 2. (*a*) Inhibition profiles of DNP-PFC by ε-DNP lysine. Cells are from spleens of mice receiving carrier-primed T cells in addition to either FACS-purified splenic ^{F}DNP-MGG (+) binding cells (stained with ^{F}DNP-MGG at 167 μg/ml), the corresponding depleted fraction (−), or whole spleen cells. Details of adoptive transfer in the Materials and Methods. ε-DNP-lysine was included in the plaquing chambers. Each point represents the geometric mean of the percent inhibition of the PFC responses of four animals compared to the geometric mean of four animals for each control group. One SE about the mean is indicated. The mean number of uninhibited PFC/spleen in these groups are given in lines 5–7 of Table III. In addition, the effect of the various concentrations of ε-DNP-lysine on a day 4 primary response to SRBC is shown. (*b*) Inhibition profiles of anti-DNP PFC by DNP-MGG. See legend of Fig. 2 *a*.

than ε-DNP-lysine.) Neither ε-DNP-lysine nor DNP_{23}-MGG inhibit anti-SRBC-PFC (see Fig. 2).

Reduction in the avidity of the DNP-PFC in recipients of cells from the fraction depleted of nearly all cells which bind ^{F}DNP-MGG at a high-staining concentration suggests that low avidity DNP-PFC precursors give rise to low avidity DNP-PFC. This is consistent with the observation that the avidity of DNP-PFC in recipients of cells from the enriched fraction appear to be slightly higher than the DNP-PFC in recipients of unfractionated spleen (see Fig. 2 *a*). The high ^{F}DNP-MGG concentration used here stained nearly all the ^{F}DNP-MGG-binding cells in the unfractionated spleen (see Tables I and II), therefore only the very low avidity precursors are expected to be absent from the enriched fraction. Thus, the higher avidity of the DNP-PFC in the recipients of cells from the enriched fraction reflects this absence.

Isolation of High Avidity ^{F}DNP-MGG-Binding Cells and Assessment of Their Precursor Activity. To determine whether high avidity ^{F}DNP-MGG-binding cells contained the precursors of high avidity PFC, normal BALB/cN splenic lymphocytes were stained at a low concentration of ^{F}DNP-MGG (10 μg/ml) to allow isolation of the highest avidity lymphocytes.

At this staining concentration, 0.2% of the lymphocytes bound detectable (by fluorescence microscopy) ^{F}DNP-MGG and the FACS-enriched fraction contained 85% ^{F}DNP-MGG-binding cells (see Table IV). The depleted fraction contained less than 0.03% fluorescent binding cells. It also should contain those low avidity binding lymphocytes which are detected at higher ^{F}DNP-MGG-staining concentrations (compare Table I).

The avidity of the DNP-PFC in recipients of the stained (high binding avidity) cells are 100-fold higher than the avidity of the DNP-PFC derived from the depleted fraction (Fig. 3). The lines in this figure show that while 75% of the DNP-PFC obtained from the enriched fraction are inhibited by 10^{-6} M ε-DNP-lysine, 10^{-4} M ε-DNP-lysine is required to inhibit the same proportion of DNP-PFC obtained from the depleted fraction. Thus, enrichment of high avidity ^{F}DNP-MGG-binding cells gives concomitant enrichment for high avidity DNP-PFC precursors.

In the recipients of cells from the depleted fraction, the avidity of the DNP-PFC were 10-fold lower than the DNP-PFC in recipients of unfractionated spleen. This contrasts with the last experiment (see Fig. 2) where the depleted fraction obtained from a population stained with a high concentration of ^{F}DNP-MGG gave rise to DNP-PFC of 300-fold lower avidity compared to unfractionated spleen. Thus, when the majority of the high avidity binding cells are removed, the depleted fraction contains the medium and low avidity DNP-PFC precursors (as well as any contaminating high avidity precursors missed in the separation) which can be removed by increasing the staining concentration. This suggests that over a wide range of avidities there is a direct correlation between the avidity of the ^{F}DNP-MGG-binding precursor cells and the avidity of the DNP-PFC they produce.

Several additional controls relevant to the entire study were included in this experiment. Isolated ^{F}DNP-MGG-binding cells did not contain anti-KLH

TABLE IV
Adoptive Primary Response of Purified High Avidity ^{F}DNP-MGG-Binding Cells

% Fluorescent cells* in fractions			No. of cells transferred ($\times 10^6$)‡				Direct anti-DNP§ PFC/spleen ($\times 10^3$)	Total anti-KLH‖ titer ($\log_2$)
Unfractionated	^{F}DNP-MGG (+)	^{F}DNP-MGG (−)	KLH-primed T¶	Unfractionated	^{F}DNP-MGG (+)	^{F}DNP-MGG (−)		
0.2	84	<0.03	—	5	—	—	9 (5–16)	3.0 ± 0.6
			7.5	—	—	—	2 (1–2)	4.7 ± 0.8
			—	—	0.05	—	0.7 (0.5–1)	2.5 ± 0.5
			—	—	—	5	8 (5–16)	2.0 ± 0
			7.5	5	—	—	30 (27–34)	12 ± 4.1
			7.5	0.05	—	—	5 (4–6)	2.7 ± 1.3
			7.5	—	**0.05**	—	20 (17–23)	3.3 ± 0.7
			7.5	—	0.05	—	3 (2–3)**	2 ± 0.5
			7.5	—	—	5	20 (17–22)	10 ± 1.8

* BALB/cN normal spleen cells were stained with ^{F}DNP-MGG at 10 μg/ml. ^{F}DNP-MGG-positive and -negative cells were isolated using the FACS as described in the Materials and Methods.

‡ Unfractionated, ^{F}DNP-MGG (+), or ^{F}DNP-MGG (−) cell populations were transferred into 600 R irradiated BAB/14 mice intravenously. All but one group of animals received 100 μg alum-precipitated DNP-KLH on day 0, 10 μg aqueous DNP-KLH on day 5, and were bled and sacrificed on day 12.

§ Geometric mean of the responses of four animals. Numbers in parentheses represent one SE about the mean.

‖ $\log_2$ anti-KLH hemagglutination titer ± SE. Each number represents the arithmetic mean of the ($\log_2$) titers of four animals.

¶ KLH-primed T cells were isolated from BAB/14 spleens by nylon wool passage and contained 95% T cells and 0.7% Ig-bearing cells as assessed by immunofluorescent staining.

** For this group of animals KLH was substituted for DNP-KLH.

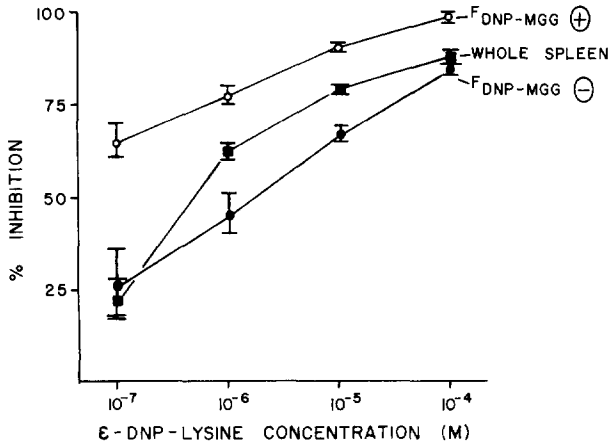

FIG. 3. Same as Fig. 2 *a* except cells were separated after staining at 10 μg/ml ^{F}DNP/MGG. The mean number of uninhibited PFC per spleen in these groups are given in lines 5, 7, and 9 of Table IV.

precursor activity, while the depleted fraction contained the same anti-KLH precursor activity as the unfractionated spleen (see Table IV). The hapten specificity of the isolated binding cells was further demonstrated since the DNP-PFC response obtained when recipients of isolated cells are challenged with KLH was 15% of the response obtained when DNP-KLH was used as the immunogen. In addition, transfer of 5×10^4 unfractionated spleen which contained 0.2% binding cells gave rise to only 5×10^3 DNP-PFC emphasizing that enrichment for ^{F}DNP-MGG-binding cells is specific for DNP-PFC precursor activity (see Table IV). To complete the demonstration that the avidity of the antigen-binding precursor cell is directly related to the avidity of the progeny PFC, low avidity ^{F}DNP-MGG-binding cells were isolated directly and shown to be the precursors of low avidity DNP-PFC.

Isolation of Medium and Low Avidity DNP-PFC Precursors. In this experiment, low avidity binding cells were differentially labeled by staining with ^{F}DNP-MGG in the presence of ϵ-DNP-lysine which blocked staining of the high avidity binding cells.

When normal BALB/cN spleen cells were stained with 55 μg/ml ^{F}DNP-MGG in the presence of a 100-fold molar excess (calculated from DNP residues) of ϵ-DNP-lysine, 0.8% of the lymphocytes bound detectable ^{F}DNP-MGG. Passage of this population through the FACS yielded an enriched population containing 90% ^{F}DNP-MGG-binding cells and a depleted fraction containing 0.03% binding cells (see Table V). The isolated binding cells were enriched 100-fold for DNP-PFC precursor activity, while the depleted fraction was depleted twofold for DNP-PFC precursor activity (see Table V).

TABLE V
Adoptive Primary Response of Purified Medium and Low Avidity ^{F}DNP-MGG-Binding Cells

% Fluorescent cells*			No. of cells transferred ($\times 10^6$)‡				Direct anti-DNP§ PFC/spleen ($\times 10^3$)
Unfractionated	^{F}DNP-MGG (+)	^{F}DNP-MGG (−)	KLH-Primed T‖	Unfractionated	^{F}DNP-MGG (+)	^{F}DNP-MGG (−)	
0.8	90	0.03	—	5	—	—	6 (5–8)
			7.5	—	—	—	4 (2–7)
			—	—	0.05	—	0.3 (0.2–0.5)
			—	—	—	5	3 (3–4)
			7.5	5	—	—	47 (41–54)
			7.5	—	0.05	—	40 (38–43)
			7.5	—	—	5	29 (26–31)

* BALB/cN normal spleen cells were stained with ^{F}DNP-MGG at 55 μg/ml in the presence of 8.7×10^{-4} M ϵ-DNP-lysine. ^{F}DNP-MGG-positive and -negative cells were isolated using the FACS as described in the Materials and Methods.

‡ Unfractionated, ^{F}DNP-MGG (+) or ^{F}DNP-MGG (−) cell populations were transferred into 600 R irradiated BAB/14 mice intravenously. All animals received 100 μg alum-precipitated DNP-KLH on day 0, 10 μg aqueous DNP-KLH on day 5, and were sacrificed on day 12.

§ Geometric mean of the responses of four animals. Numbers in parentheses represent one SE about the mean.

‖ KLH-primed T cells were isolated from BAB/14 spleens by nylon wool passage and contained 93% T cells and 1% Ig-bearing cells as assessed by immunofluorescent staining.

The data in Fig. 4 show that the avidity of the DNP-PFC obtained from the isolated medium and low avidity binding cells are 100-fold lower than the avidity of the DNP-PFC obtained from the depleted fraction. While 10^{-5} M ϵ-DNP-lysine inhibited 60% of the DNP-PFC obtained from the enriched fraction, only 10^{-7} M was required to inhibit the same proportion of DNP-PFC obtained from the depleted fraction.

The avidity of the DNP-PFC obtained from enriched fraction in this experiment were slightly lower than the avidity of the DNP-PFC obtained from the unfractionated spleen, which suggests that under these staining conditions,

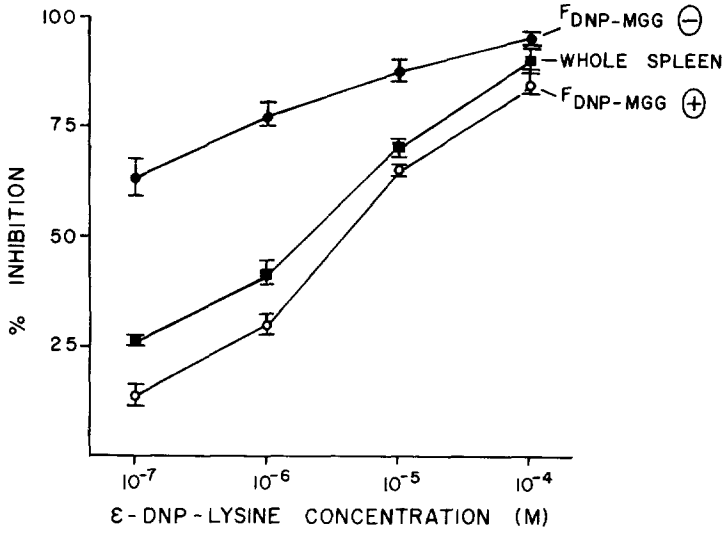

FIG. 4. Same as Figure 2 *a* except cells were separated after staining at 55 μg/ml ^{F}DNP-MGG in the presence of 8.7×10^{-4} M ϵ-DNP-lysine. The mean number of uninhibited PFC per spleen in these groups are given in lines 5–7 of Table V.

the medium and low avidity DNP-PFC precursors are stained while staining of the highest avidity precursors was blocked by ϵ-DNP-lysine.

Discussion

The concept of clonal selection (28) rests on two basic assumptions: (*a*) precursors of antibody-forming cells are antigen-binding cells, and (*b*) antigen receptors on the precursors have the same specificity and affinity as the antibody secreted by the progeny plasma cells. Although much of the data accumulated in cellular immunology is consistent with the predictions of clonal theory, there have been few studies which provide direct evidence to validate these basic assumptions.

To demonstrate that the precursors of antibody-forming cells are antigen-binding cells, Wigzell and Mäkelä (29) passed suspensions of primed or unprimed spleen cells through antigen-coated columns. With this method, they specifically depleted the precursors from the column effluent population but were unable to recover cells enriched for precursor activity from the column. Ada and Byrt (30) also showed that specific precursors are depleted from spleen cell populations when allowed to bind highly radioactive antigen ("suicide" experiments). These experiments gave strong support for the role of antigen-

binding cells as precursors but the question still remained open because of the inability to isolate these cells and directly test their function.

The development of the FACS has now allowed the isolation of functional antigen-binding cells. In a previous publication, we showed that the KLH-binding cells isolated from KLH-primed spleen contained all the anti-KLH precursors present in intact spleen (5). We therefore firmly established that the precursors of antibody-forming cells in primed spleen are antigen-binding cells.

In this work we have extended this conclusion to antigen-binding cells in unprimed animals. We have shown that isolated DNP-binding cells are the precursors of anti-DNP antibody-secreting cells. Moreover, we have shown that the avidity of the antibody produced by the antibody-secreting cell reflects the avidity of the receptor on the antigen-binding precursor cell.

In the experiments reported here, high avidity DNP-binding cells gave rise to predominantly high avidity DNP-PFC. Isolation and transfer of medium and low avidity binding cells gave rise to medium and low avidity DNP-PFC. In both cases, transfer of the complementary unstained populations (i.e., the "depleted" fractions) gave rise to DNP-PFC with avidities in the range expected for those precursors intentionally left unstained.

The determination of DNP-PFC avidities is based on their inhibition by ϵ-DNP-lysine as described by Andersson (3) in 1970. Hapten inhibition of PFC has been shown to be a valid measure of antibody avidity in other systems. Yamada et al. (31) have shown that mouse myeloma (MOPC-315) cells, which secrete homogeneous IgA molecules with a uniform binding constant for DNP, form DNP-specific plaques. All of the DNP-PFC were sharply inhibited over a very narrow concentration range of ϵ-DNP-lysine included in the plaquing assay. 50% of the DNP-PFC were inhibited by a concentration of ϵ-DNP-lysine which closely approximated the reciprocal of the binding constant of the myeloma protein.

Although these results indicate the possibility of avidity, or in fact affinity, measurements at the cellular level, certain restrictions to the use of hapten inhibition for avidity determinations merit consideration here. First, it may be used (uncorrected) to determine avidity only when there is a relatively constant rate of antibody secretion among PFC in a given experiment, i.e., when plaque size is relatively constant. Since the avidity measured by this method is a weighted average between the local antibody concentration and the true avidity of the antibody secreted, large differences in rates of antibody secretion will cause apparent avidity differences between two PFC secreting identical antibody molecules. Such differences have been shown experimentally with PFC from the B-cell clone E9 which secretes a homogeneous immunoglobulin specific for DNP when large variation in uninhibited plaque size was observed (~10-fold) (32). In our experiments, the variation in uninhibited plaque size was small (~twofold). Therefore, it is likely that the major parameter measured in these experiments is relative avidity of anti-DNP antibody rather than the amount secreted per DNP-PFC.

Secondly, if IgG and IgM PFC are present in the same experiment, the hapten concentrations giving equivalent inhibitions might well be quite different due to the differing valencies of the different antibodies. In these experiments, as will be further discussed below, only IgM PFC are produced.

Thirdly, comparison of avidities for PFC between experiments may be made only when the extent of hapten conjugation to indicator erythrocytes is kept constant. Pasanen and Mäkelä (33) have shown that the ability to inhibit PFC with free hapten decreases as the extent of hapten conjugation to indicator cells increases. Therefore, while comparison of PFC avidity is valid within a given experiment, it must be considered a relative value when comparing experiments unless the hapten conjugation is strictly controlled between experiments. This restriction applies to the data we have reported since each experiment was performed with newly conjugated erythrocytes which may have varied somewhat with respect to the extent of hapten conjugation. Therefore, the data is discussed in terms of the avidities of DNP-PFC from the unfractionated populations in each experiment.

Despite this reservation, there is at least a rough correlation between average avidities measured in different experiments. The average avidities of DNP-PFC obtained from adoptive secondary transfers (i.e., transfer of DNP-KLH-primed spleen) measured in other experiments are 100-fold higher than the average avidities of the DNP-PFC which we find here in an adoptive primary transfer[5] (high avidity PFC obtained in adoptive secondary transfers are predominantly due to IgG antibody, whereas the PFC in these adoptive primary transfers are virtually all due to IgM antibody). This difference in avidities is consistent with higher affinity antibody found in a secondary response (1) and suggests that the hapten inhibition method may be used for comparisons between experiments when there are large avidity differences.

Very few, if any, IgG DNP-PFC are found in the adoptive primary recipients in the experiments reported here, although supplementation of an unprimed precursor population with carrier-primed cooperator cells has been shown to favor the production of IgG PFC in other systems (34, 35). It may be that the numbers of IgG DNP-PFC would have been larger had there been a larger interval between antigen boost and day of assay for PFC. Using a similar adoptive primary transfer system, the peak IgG anti-DNP serum titer occurred at day 30 after transfer, while at day 12 the anti-DNP titer was due completely to IgM antibody.[6]

The absolute primed T-cell dependence of this adoptive primary DNP response both with unfractionated spleen cells and the isolated antigen-binding cells is remarkable in view of the wholly IgM response (at 12 days). This absolute dependence is manifest even when only the highest avidity DNP precursors are used with all the remaining normal spleen cells present, except for low and medium avidity DNP-binding cells hence precursors. This may provide an exquisitely sensitive system for assay of active T cells or T-cell substitutes. The recipients are boosted with rather large amounts of DNP-KLH for the presumed relatively few high avidity precursors. Any explanation of T-cell dependence invoking aid to better antigen binding by precursors would be least applicable, one would expect, to these high avidity cells. It will be interesting to see whether continued stimulation of these precursors will lead to production of IgG DNP-PFC and with higher avidities or whether some suppression of these indirect PFC is occurring.

[5] Metzler, C. M. Unpublished observation.

[6] Segal, S. Personal communication.

Summary

Cells binding DNP groups conjugated to fluoresceinated mouse gamma globulin (^{F}DNP-MGG) were isolated from spleens of unprimed mice using a fluorescence-activated cell sorter (FACS). The isolated cells were specifically enriched at least 100-fold for anti-DNP precursor activity in an adoptive transfer assay as compared to unfractionated spleen. The fraction depleted of binding cells, although depleted of anti-DNP precursor activity, responded as well as unfractionated spleen when assayed for anticarrier (keyhole limpet hemocyanin [KLH]) precursor activity.

High avidity binding cells were stained using low concentrations of ^{F}DNP-MGG. Medium and low avidity binding cells were stained using high concentrations of ^{F}DNP-MGG in the presence of free hapten which selectively blocked staining of the high avidity binding cells. Cells were supplemented with an excess of carrier-primed (KLH), nylon-purified splenic T cells and transferred to irradiated recipients. DNP-KLH was given at transfer and 5 days later. The anti-DNP plaque-forming cell (DNP-PFC) response and the avidities of the DNP-PFC in the irradiated recipients were measured by hapten inhibition of direct PFC plaque formation 12 days after transfer. At this time, very few indirect PFC were found.

There was a positive correlation between the avidity of the DNP-binding cells and the avidity of the anti-DNP antibody secreted by their progeny. High avidity DNP-binding cells gave rise to predominantly high avidity anti-DNP-PFC. Medium and low avidity binding cells gave rise to medium and low avidity DNP-PFC.

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