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Cell Separation on Affinity Columns: The Isolation of Immunospecific Precursor Cells from Unimmunized Mice

(lactoside hapten/lymphocyte receptors/immunology)

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ABSTRACT **We have used affinity columns to isolate from the spleens of unimmunized mice a population of lymphocytes that specifically bind a lactoside hapten. These cells are able to initiate an antibody response to azophenyl-β-lactoside when transferred to appropriate irradiated recipients.**

Considerable evidence supports a clonal selection theory of antibody formation (1). It is generally believed that normal, unimmunized vertebrates contain many diverse populations of lymphocytes that exhibit antibody-like receptors on their surfaces and that a given antigen stimulates only selected precursor cells to proliferate and to give rise to antibody-producing cells of corresponding specificity. For secondary lymphoid tissues, estimates of the frequency of precursor cells specific for a multideterminant antigen (e.g., foreign erythrocytes or polymeric flagellin) are in the range of 5×10^{-4} to 10^{-5} (2). To investigate cellular differentiation in the immune system, it should be advantageous to obtain pure populations of lymphocytes with a common receptor specificity that can be induced to antibody response or tolerance to a defined antigenic determinant.

We report here the specific purification of precursor cells for a primary antibody response to azophenyl-β-lactoside (N_2Phlac). The cells have been isolated from the spleens of unimmunized mice by filtration of cell suspensions through affinity columns of large polyacrylamide beads to which N_2Phlac groups are covalently attached (3, 4). Specifically-binding cells were recovered by elution with a lactoside hapten, by use of essentially the same procedures that have previously permitted us to prepare highly enriched populations of anti-N_2Phlac antibody-producing cells from the spleens of immunized animals (4). The presence of a high proportion of anti-N_2Phlac-specific precursors in the cells purified from normal spleens has been demonstrated by transferring and testing the cells in irradiated mice. Since antihapten antibody precursor cells cannot respond productively to antigen without the cooperation of thymic-derived helper cells (5), a source of helper cells was provided in host animals by priming them with antigen 1 month before irradiation and cell transfer.

Abbreviations: N_2Phlac, azophenyl-β-lactoside; KLH-N_2Phlac, keyhole limpet hemocyanin-azophenyl-β-lactoside, PFC, plaque forming cells; BSS, balanced salt solution of Mishell and Dutton (7).

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MATERIALS AND METHODS

Preparation of N_2Phlac-Affinity Beads. N_2Phlac-affinity beads were prepared as before (3), except that the time of reaction of the Bio-Gel with hydrazine was reduced to 1.25 hr to limit the number of amide groups modified (6) and to better preserve the structural integrity of the beads. Mechanical stirring was avoided in all preparative procedures.

Specific Cell Purification. Single-cell suspensions were prepared from pooled spleens of 4- to 6-months-old unimmunized Balb/c mice. Cells were washed and resuspended in balanced salt solution (BSS) (7) without glucose immediately before addition to columns. Column procedures followed those described for column preparation of pure populations of anti-N_2Phlac-specific lymphocytes (4). In the present experiments, we used several small columns, each containing 2.5 ml of N_2Phlac-affinity beads. About 5×10^8 cells, at a concentration of 8×10^6 cells per ml, were passed through each column, which was then washed with 5 ml of BSS. Columns were then equilibrated with buffer (pH 7.2) containing *p*-aminophenyl-β-lactoside; cells were eluted from each column in a 4 ml volume of BSS, 15 μM in the lactoside hapten, and containing 0.1% glucose. When the cells were to be used in transfer experiments, irradiated (1500 R) normal thymocytes were added to the eluting buffer (pH 7.2) at a concentration of 10^7 cells per ml. This was necessary so that the specifically eluted cells could be recovered efficiently during two centrifugation cycles required for concentrating the cells and diluting out the hapten.

Cell Counts. The number of cells in the eluted suspensions from normal spleens was too low to permit counting by conventional means. Therefore, a measured volume of a hapten-eluted cell suspension was passed through a 13-mm Swinnex Millipore filter, which was then stained with hematoxylin and eosin and mounted by standard histological procedures. Cells were counted under a microscope at ×160 magnification. When cells from immunized mice were recovered after specific purification on N_2Phlac-affinity columns, the number of eluted cells was high enough to permit counting with a Coulter Counter, model A. For purposes of comparison, some of these preparations were also counted by the Millipore filtration procedure.

Cell Transfers. Balb/c mice to be used as recipients in transfer experiments were primed 27–31 days previously with a foot-pad injection of 50 μg of KLH-N_2Phlac antigen (3) in

complete Freund's adjuvant. Recipients were exposed to 750 R 4–6 hr before intravenous injection with 0.25 ml containing the appropriate test cell suspension, 2×10^7 irradiated thymocytes, and 50 μg of keyhole limpet hemocyanin-azophenyl-β-lactoside (KLH-N_2Phlac) antigen. Since in transfer experiments the anti-N_2Phlac response increases to day 8, and remains at about the same level through day 12, recipient spleens were assayed for antibody-producing cells 10 days after transfer.

Assay of Plaque-Forming Cells (PFC). The hemolytic plaque technique (8) was used to detect cells synthesizing antibodies. The net anti-N_2PHlac response was obtained by subtraction of the number of plaques against unmodified sheep erythrocytes from the values obtained with N_2Phlac-coupled sheep erythrocytes (3). Both direct and indirect plaques were scored. Since the anti-mouse gammaglobulin used to develop the indirect plaques only slightly inhibited direct (IgM) plaque formation, the number of IgG PFC was determined by subtraction of the plaque number obtained in the absence of developing serum.

RESULTS

Cell counts of specifically purified populations

The number of cells recovered in hapten-eluates when normal spleen cell suspensions were applied to N_2Phlac-affinity columns was highly reproducible, about 3000 per 10^8 passed cells, with a range of 1000–4000 per 10^8 cells in eight separate experiments. This was in contrast to counts of hapten-eluted cells when spleens were obtained from mice primed once with the KLH-N_2Phlac antigen, 2–6 weeks previously: the number of purified cells from primed mice in six separate experiments ranged from 50,000 to 200,000 per 10^8 cells passed through the columns.

The purified cells from normal spleens appear by light microscopy to resemble small lymphocytes.

Transfer Experiments. In Table 1, we have pooled the results of two essentially indistinguishable transfer experiments. Irradiated primed hosts were divided into three groups: each mouse in group 1 received no spleen cells; each mouse in group 2 received 6×10^3 hapten-eluted cells purified from normal spleens; each mouse in group 3 received 2×10^7 unfractionated normal spleen cells. Some mice in group 1 showed precursor activity that survived irradiation: the geometric means for the group were 5 IgM and 425 IgG anti-N_2Phlac PFC per spleen. Above this background, group 2 gave significantly elevated IgM and IgG responses.‡ The geometric means for anti-N_2Phlac PFC per spleen were 62 IgM and 2040 IgG for mice given eluted cells and 22 IgM and 3154 IgG for mice given the 3000-fold larger number of unfractionated normal spleen cells. Three of the four highest individual responders were recipients of the N_2Phlac-specific cells.

Comparison of background levels of anti-sheep erythrocyte PFC in the three groups showed an average splenic content of 415 among the mice that received 2×10^7 unfractionated spleen cells, while the levels for mice in groups 1 and 2 were very low: 29 and 33, respectively. A similar contrast was observed in the average number of cells recovered per spleen: The spleens of mice given only irradiated cells or eluted spleen cells were many times smaller than those that received the unfractionated cells (Table 1).

‡ By Wilcoxon Rank Sum Test, the increased IgM response is significant at 0.01, one-sided, and the IgG at 0.025, one-sided; for IgG, the same result is obtained by a conventional *t* Test on log PFC responses. Combining the IgM and IgG information as independent evidence, one obtains a *P* value of 0.006. [Fisher, R. A. (1954) in *Statistical Methods for Research Workers* (Hafner Publishing Co., New York), 12th Ed., p. 99.]

TABLE 1. *Anti-N_2Phlac PFC spleen responses* of irradiated mice†*

	Group 1	Group 2	Group 3
	6(17)	0(128)	80(200)
	0(45)	13(424)	310(1100)
	0(117)	15(610)	550(2250)
	0(180)	45(930)	2480(2800)
	142(480)	405(1200)	0(3000)
	0(480)	150(2130)	0(3540)
	52(890)	108(2260)	135(3660)
	0(1000)	30(3760)	260(4500)
	0(1375)	120(3850)	0(5700)
	48(3400)	885(7800)	0(6800)
	0(5380)	95(12,900)	0(20,400)
		170(18,700)	
Geometric mean:	5(425)	62(2040)	22(3154)
Cells per spleen‡:	8.1×10^6	1.3×10^7	7.4×10^7
Anti-sheep erythrocyte PFC per spleen‡:	29	33	415

* Unenclosed figures represent IgM responses; those in parentheses are the IgG responses.

† Mice were primed with 50 μg of KLH-N_2Phlac 27–31 days before irradiation and cell transfer. Each mouse received 2×10^7 irradiated thymocytes. Those in group 2 received in addition 6×10^3 normal spleen cells eluted with N_2Phlac hapten from affinity columns. Those in group 3 received 2×10^7 unfractionated normal spleen cells.

‡ Geometric mean values.

DISCUSSION

These results provide strikingly direct evidence for the clonal selection theory of antibody formation, showing that precursor activity for response to a particular antigen resides in a small number of selected cells with corresponding cell-surface receptor specificity. A few thousand specifically purified cells from unimmunized mice give an antihapten response virtually equivalent to that produced by about 10^7 unfractionated normal cells.

Exploration of the kinetics and other parameters of the response of the purified cells is limited by the numbers of spleen cells that can be processed on columns and prepared for a given transfer experiment, particularly in view of the low frequency of precursor cells in a normal population. The observed enhancement of the capacity for anti-N_2Phlac response attributable to the hapten-eluted cells is, however, clearly significant and is not due to nonspecific stimulatory factors. In contrast to 2×10^7 unfractionated cells, the purified cells do not affect the background level of PFC to sheep erythrocytes or markedly elevate the extent of cell repopulation in irradiated spleens.

The procedure of washing the column free of nonbound cells followed by specific hapten elution assures recovery of a virtually pure population of anti-N_2Phlac specific lympho-

cytes. That the level of inadvertently collected, nonspecific cells must be extremely low is illustrated by the fact that priming with antigen results in as high as 70-fold increases in the number of specifically purified lymphocytes. Purity is further indicated by the infrequent occurrence of contaminating erythrocytes, although the latter comprise more than 50% of the cells in spleen suspensions. Over 80% of the cells purified from normal mouse spleens by means of the N_2Phlac affinity columns can form specific rosettes with N_2Phlac-modified sheep erythrocytes (personal communication from P. Truffa-Bachi).

We cannot tell from these experiments precisely how many specific lactoside-binding lymphocytes are present in unimmunized mouse spleens, nor how high a proportion of the recovered cells can function as immunospecific precursors. The ratio of recovered cells to the total number put on columns is 3×10^{-5}; this is consistent with expectations that the frequency of normal spleen cells that specifically bind a simple hydrophilic hapten should be considerably lower than the range of 3×10^{-3} to 2×10^{-4} that has been reported in studies on the binding of complex radioactive or fluorescent antigens (2). It is probable, however, that our yield of anti-N_2Phlac specific cells in the pure populations is only partial. For PFC, where column yields can be quantitatively evaluated, our recoveries range from 10–70% (4).

The 6000 eluted cells given to individual irradiated hosts were the purified product obtained from 2×10^8 normal spleen cells. Determination of the actual number of viable, functionally competent, anti-N_2Phlac precursors would depend in part on test systems in which helper cells are not limiting, and, perhaps, on the development of *in vitro* techniques where the homing capabilities of the transferred purified cells would not be a factor. In the present experiments, our antigen has been an exhaustively azo-coupled KLH-N_2Phlac that provokes an excellent anti-N_2Phlac response in mice and makes feasible the investigation even of primary immunizations. However, the azoprotein carrier no longer bears significant antigenic relationship to native hemocyanin; thus, we could not successfully generate helper cells in host mice by priming for the protein carrier alone, thereby restricting the antihapten response exclusively to transferred cells. While the results reported here indicate that some helper cell function survives irradiation, separate transfer experiments with primed donor cells indicate that the irradiated hosts are a poor source of helper cells.

Methods for the depletion of selected immunocompetent cells have already contributed significant, though indirect, evidence of the role of antigen-binding cell-surface receptors (9). Our present findings, and the earlier purification of anti-N_2Phlac PFC (4), establish that cells at various stages of differentiation can be isolated directly on the basis of receptor specificity in highly purified populations with retention of functional capacity. The appropriate use of affinity columns, or similar methods currently in development (10), should provide the possibilities for examination of specific interactions between antigen and receptor on lymphocyte membranes.

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