

Binding of Thymus- and Bone Marrow-Derived Lymphoid Cells to Antigen-Derivatized Fibers

(mouse spleen/nylon fibers/antiserum/cytotoxicity/fluorescence)

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ABSTRACT Thymus-derived lymphocytes (T cells) and bone marrow-derived lymphocytes (B cells) from mouse spleens bind specifically to antigen-derivatized nylon fibers. The fiber-bound population consisted of about 60%-70% B cells and 30% T cells as determined by cytotoxicity, fluorescence, and antibody-complement binding assays. Essentially all fiber-bound cells were viable and could be accounted for as T or B cells. Enriched populations of T or B cells could be isolated on the fibers by destruction of one or the other cell type with the appropriate antiserum plus complement. T or B cells could also be fractionated according to their relative affinity (or avidity) for a given antigen.

In previous reports, we have described the fractionation of specific antigen-binding cells from lymphoid cell populations of immunized and unimmunized animals by specific attachment to antigen-derivatized fibers (1, 2). Although the isolated lymphoid cells all have specificity for the chosen antigens, it is likely that they are heterogeneous in their function and degree of differentiation. The most conspicuous functional heterogeneity among lymphocytes is reflected by the presence in the population of thymus-derived lymphocytes (T cells) and bone marrow- or bursa-derived lymphocytes (B cells) (3). Additional distinctions must be made between thymocytes and competent T cells. Similarly, B cells include several functional classes, notably antigen-sensitive B cells that can differentiate into secreting cells when triggered by antigen, and the secreting cells or plaque-forming cells themselves.

Our studies (1, 2) have indicated that specific antigen-binding cells isolated by fiber fractionation do not include either thymocytes or plaque-forming cells. In the present paper, we provide evidence that antigen-derivatized fibers bind both T and B cells. The cells can be fractionated according to their relative avidities* for antigen and, by the use of appropriate antisera, enriched populations of T or B cells with specificity for a given antigenic determinant can be obtained.

MATERIALS AND METHODS

Cell Suspensions. Cell suspensions were prepared at 4° from spleen, thymus, or bone marrow of Balb/c mice (Jackson

Abbreviations: BSA, bovine-serum albumin (recrystallized, Armour Pharmaceutical; Kankakee, Ill.); EAC, sheep erythrocyte-antibody-complement complex; FBC, fiber binding cells; MEM, minimal essential medium, Eagle's, with Earle's Balanced Salts, without bicarbonate; T cells, thymus-derived lymphoid cells; B cells, bone marrow- or bursa-derived lymphoid cells.

* We will use the term avidity to designate the apparent affinity of a cell, with its many individual antigen-binding receptors, for a particular antigen molecule. In the case of a monovalent antigen, the avidity of the cell is assumed to be equivalent to the affinity of

Laboratories, Bar Harbor, Maine). Aggregated material was removed by low-speed centrifugation, and the cells were washed three times in Minimal Essential Medium without NaHCO₃ (MEM, Microbiological Associates, Bethesda, Md.).

Immunization. Mice were injected intraperitoneally at monthly intervals with 200 µg of Dnp-hemocyanin adsorbed onto bentonite (4). Spleens were removed 4-6 days after the second or third injection.

Preparation of Anti-θ Serum. Anti-θC₃H antibodies were prepared by the method of Reif and Allen (5). AKR/J (H-2k) mice were injected intraperitoneally with 10⁷ C57B1/6J (H-2b) or C₃H/HeJ (H-2k) mouse thymocytes at weekly intervals for 10 weeks. Blood was collected from the retro-orbital sinus 6 days after the sixth and each succeeding injection. In the presence of complement, this antiserum was specifically cytotoxic for T cells (6) from Balb/c (H-2d) mice. At a dilution of 1:10, the antiserum killed over 90% of Balb/c thymocytes and 30%-40% of Balb/c spleen cells, but less than 5% of Balb/c bone marrow cells or AKR thymocytes. Agarose-adsorbed guinea pig complement (Grand Island Biological Co., Grand Island, N.Y., lyophilized and freshly reconstituted) was used at a final dilution of 1:10. These concentrations of antibody and complement were optimal for each of the cytotoxicity assays in this paper.

Preparation of Antibodies to Immunoglobulin. Rabbits were immunized in their footpads with 2 mg of purified mouse immunoglobulin in complete Freund's adjuvant and were restimulated subcutaneously at 1-month intervals with 1 mg of antigen in saline. Serum was obtained 7 days after each injection and contained anti-kappa, anti-gamma, and a trace anti-mu chain antibodies. For fluorescent staining, the immunoglobulin fraction was isolated and labeled with fluorescein (7).

Fiber Fractionation of Spleen Cells. Nylon fibers were strung in dishes, derivatized with hapten-bovine-serum albumin (BSA) conjugates (at 0.25 mg/ml), BSA (5 mg/ml), Limulus hemocyanin (2.5 mg/ml), or concanavalin A (0.25 mg/ml), and incubated with cells in MEM containing 20 µg/ml of DNase at 4° (1, 2). Binding to the Dnp-BSA fibers was over 90% inhibitable by 300 µg/ml of Dnp-BSA, or 250 µg/ml of anti-mouse immunoglobulin, but was not inhibited by several other hapten-BSA conjugates. The isolated cells were more than 95% viable as judged by trypan-blue exclusion.

Cytotoxicity Assay of Fractionated Cells. After all unbound cells were washed away, the cytotoxic effect of anti-θ serum and anti-mouse immunoglobulin serum on fractionated cells

TABLE 1. Cytotoxic effect of anti- θ serum on spleen cells bound to Dnp-BSA-derivatized fibers

	Balb/c		Balb/c	AKR
	Unimmunized	Immunized	(Nu/Nu) Unimmunized	Unimmunized
Number of FBC*	377	958	185	475
Percentage of FBC† killed by anti- θ	25-40	30-41	4-6	1-4
Percentage of FBC killed by mouse serum	0	1	3	2

* Expressed as number of cells bound to the edges of a 2.5-cm fiber segment.

† Range of four experiments.

were determined on cells bound to the fibers and cells removed from the fibers by plucking (1).

Fiber-bound cells (FBC) were incubated with a 1:10 dilution of anti- θ serum or anti-mouse immunoglobulin serum in MEM at 37° for 45 min. The serum was then washed away and the cells were incubated with a 1:10 dilution of agarose-absorbed guinea pig complement for 30 min at 37° with gentle shaking. In most experiments, when strong antiserum and complement were used the cells bearing the target antigen were severely damaged and no longer remained bound to the fibers; with less potent reagents, some dead cells were not released. Cytotoxicity was determined by counts of the number of viable bound cells before and after treatment with complement.

In a separate cytotoxicity assay, cells were released from the fiber by plucking into 3 ml of MEM containing either 10% anti- θ serum or mouse serum, and 10% complement. This suspension was incubated at 37° for 1 hr and then cytocentrifuged for 2 min at 1500 rpm (leaving the cells wet) onto a microscope slide. A drop of 0.15% trypan blue in 0.15 M NaCl was added, and the stained and unstained cells were scored.

Fluorescent Labeling of Fractionated Cells. To detect immunoglobulin receptors, fiber-bound cells were incubated *in situ* at 25° for 30 min with a 200 μ g/ml solution of rabbit anti-mouse immunoglobulin that had been labeled with fluorescein. After washing, the cells were released from the fibers by plucking and cytocentrifuged for 2 min at 1500 rpm onto a glass slide for observation. Stained cells showed bright caps of fluorescent material.

A fluorescent sandwich procedure was used to detect cells with the θ marker. Fiber-bound cells were treated with unlabeled rabbit anti-mouse immunoglobulin to mask cell surface immunoglobulin, washed, and incubated with a 1:50 dilution of anti- θ serum. The cell-bound anti- θ was then stained with a 200 μ g/ml solution of anti-mouse immunoglobulin that had been labeled with fluorescein. Stained cells showed bright patches of fluorescent material.

Formation of EAC Rosettes by Fiber-Bound Cells. Sheep erythrocyte-antibody-complement complexes (EAC) were prepared as described by Bianco *et al.* (8). Spleen cells from mice immunized with Dnp-hemocyanin were fractionated with Dnp-BSA-derivatized fibers and incubated with 4%

EAC in MEM with gentle shaking for 30 min at 37°. The unbound EAC were washed away, and the percentage of cells that formed EAC rosettes was determined by direct microscopic observation of the fibers. The rosettes were stable for more than 1 hr at room temperature.

Fractionation of Cells According to Their Avidity for Dnp. Incubation of spleen cells with Dnp-BSA-derivatized fibers in the presence of various concentrations of Dnp-BSA or ϵ -Dnp-lysine (*N*-dinitrophenyl- ϵ -L-lysine monohydrochloride, Mann Research Laboratory, New York, N.Y.) permitted fractionation of the cells with respect to their avidity for the soluble inhibitor (2). With cells from immunized mice, the inhibition increased with inhibitor concentrations from 0.002 to 300 μ g/ml. After fractionation, the inhibitor and unbound cells were washed away, the bound cells were treated with antiserum and complement, and the numbers of T and B cells were counted as above.

RESULTS

The cytotoxic effect of anti- θ C₃H serum on Balb/c spleen cells that were specifically bound to Dnp-BSA-derivatized fibers is shown in Fig. 1 and Table 1. The cytolytic activity of the anti- θ serum could be completely absorbed by brain homogenates or thymocytes from Balb/c mice. About one-third of the fiber-binding spleen cells from both immunized and unimmunized mice were specifically killed and released from fibers by treatment with anti- θ serum and complement, but not by normal mouse serum and complement. AKR spleen cells and spleen cells from nude Balb/c mice, which are T-cell deficient (9), were not affected by the anti- θ antiserum.

The population of cells that had been removed from the fibers after specific fractionation included 20-50% T cells (Table 2). As controls for the effect of fiber fractionation on the cytotoxicity assay, cells from Balb/c spleen, thymus, and

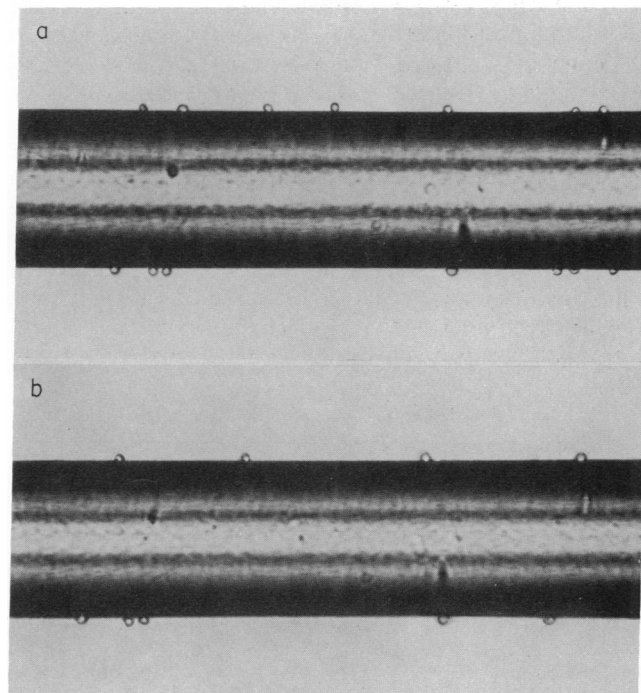


FIG. 1. Spleen cells bound to the same segment of a Dnp-BSA-derivatized fiber before (a) and after (b) treatment with anti- θ serum and complement.

TABLE 2. Cytotoxic effect of anti- θ serum on Balb/c cells removed from fibers after fractionation

Organ	Spleen	Spleen	Spleen	Thymus	Bone marrow
Immunization	—	Dnp-hem-ocyanin	—	—	—
Fiber	Dnp-BSA	Dnp-BSA	Con A*	Con A	Con A
Percentage of cells killed by anti- θ †	20-53	20-42	26-38	>89	0-5

* Concanavalin A.

† Percentage killing above control values (8-14%) with mouse serum and complement. Values represent the range of four experiments.

bone marrow were bound to fibers with concanavalin A, which binds most of the nucleated cells from these organs. After they were removed from the fibers and incubated with normal mouse serum and complement, the viability of these cells was more than 85%. With anti- θ treatment, thymocytes from concanavalin A-derivatized fibers were almost completely destroyed, whereas the viability of bone marrow cells was not diminished. About 32% of spleen cells removed from concanavalin A-derivatized fibers were killed by anti- θ serum. These results with concanavalin A-bound cells are compatible with the percentages of T cells found (6, 10) in unfractionated cell suspensions of these organs.

Antibodies to immunoglobulins are cytotoxic for B cells, but not for T cells (11); 60% of the cells bound to Dnp-BSA-derivatized fibers were killed by anti-mouse immunoglobulin plus complement (Table 3). The results shown in Table 3 also indicate that the cytotoxic effects of anti-immunoglobulin serum and anti- θ serum on cells bound to a Dnp-BSA fiber are additive and nonoverlapping. Sequential treatment with both antisera, in either order, killed and removed 97% of the fiber-fractionated cells. Thus, practically all of the bound cells appear to be either T or B lymphocytes.

B cells have receptors for antigen-antibody-complement complexes (12). When sheep erythrocytes (8) are used as the antigen in these complexes, visible "EAC rosettes" are formed as a result of their binding to B cells. Since T cells do not have these receptors, cells bound to fibers can be identified as B cells by this property (Fig. 2). With spleen cells from mice

TABLE 3. Cytolysis of spleen cells bound to Dnp-BSA derivatized fibers after sequential treatment with anti- θ and anti-mouse immunoglobulin

First treatment		Second treatment		% Cells killed by both treatments
Serum	% Cells killed*	Serum	% Cells killed*	
Anti- θ	33	Anti-immunoglobulin	64	97
Anti-immunoglobulin	55	Anti- θ	42	97
Mouse serum	5	Rabbit serum	3	8

* Expressed as % of original FBC killed by each treatment.

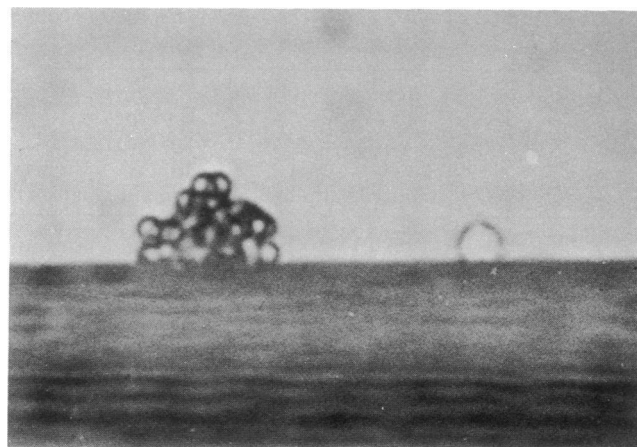


FIG. 2. Section of a fiber derivatized with Dnp-BSA showing one cell that binds EAC and one that does not.

immunized with Dnp-hemocyanin, 66-70% of the cells bound to Dnp-BSA fibers formed EAC rosettes.

The percentages of T and B cells in cell populations fractionated according to several different antigenic specificities are shown in Table 4. Although the number of bound cells varied considerably, all the antigen-specific populations included essentially the same proportion of T and B cells.

Fluorescence microscopy was also used to permit the detection of T and B cells (13) after removal from the fibers (Table 5). A large proportion of the fiber-fractionated cells treated with fluorescein-labeled anti-immunoglobulin showed intense fluorescent caps characteristic of B cells. When an indirect fluorescent stain for the θ antigen was used, a significant proportion of T cells was also found among the cells isolated from the antigen-derivatized fibers, although the values were about 10% less than those determined by cytotoxicity tests. The intensity of fluorescent staining of T cells was lower for the specifically isolated antigen-binding cells than for whole spleen cell preparations; it is possible that for this reason some T cells were not detected.

We have shown that spleen cells can be fractionated according to their avidity for Dnp by addition of increasing amounts of soluble Dnp₁₀-BSA or ϵ -Dnp-lysine to the cell suspension during incubations with Dnp-BSA-derivatized fibers (2). Once bound, cells cannot be removed from the fibers by soluble inhibitor (1). Under these conditions, cells with high avidities for the inhibitor are prevented from binding at lower inhibitor concentrations than cells with low avidities. Thus, as the concentration of inhibitor increases, the percentage of inhibition increases, and the cells that do bind to the fibers have a lower average avidity. After this procedure was used, fractionated lymphocytes were assayed for T and B cells with anti- θ serum or anti-immunoglobulin serum. The difference between the inhibition curve for T cells and B cells (Fig. 3a) suggests that, under the conditions of our experiments, T cells have a higher apparent affinity or avidity for the polyvalent inhibitor Dnp₁₀-BSA than do B cells. When ϵ -Dnp-lysine was used as an inhibitor (Fig. 3b), however, this difference was not observed.

DISCUSSION

Fiber fractionation of immune cell populations has been used to isolate specific antigen-binding cells against various anti-

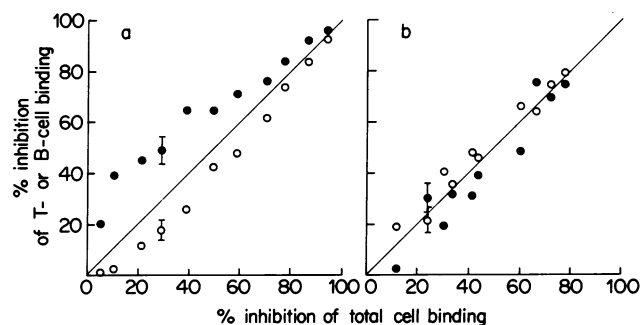


FIG. 3. Percent inhibition of T cell (●) and B cell (○) binding to Dnp-BSA-derivatized fibers as a function of the percent inhibition of total cell binding. Inhibition was obtained with different amounts of a) Dnp₁₀-BSA or b) ϵ -Dnp-lysine. As described (2), the percent inhibition of total cell binding increased from 0 to 90% as the concentrations of either inhibitor increased from 0 to 300 μ g/ml. T and B cells were identified by cytolytic treatment of the total bound cell population with anti- θ or anti-mouse immunoglobulin sera. The cells were obtained from the spleens of mice 5 days after secondary immunization with Dnp-hemocyanin. Error bars represent the standard deviation from the average values obtained in four experiments. If there were no differences in the binding behavior of T and B cells, the points would fall on the 45° line.

gens (2). Unlike the specific solid phases used in other fractionation methods (14, 15), antigen-derivatized fibers bind both T and B cells. By use of the appropriate antiserum and complement on the fiber-fractionated cells, either T or B cells can be removed from the fibers: T cells can be isolated by treating the fiber-bound cells with anti-immunoglobulin and B cells by treatment with anti- θ (Fig. 1). Alternatively, T or B cells can first be isolated by various methods (16), and can then be fiber-fractionated with respect to a given antigenic specificity.

Thymus-derived lymphocytes bind antigens as determined by the rosette assay (17, 18), by "antigen-suicide" experiments (19), and now by fiber fractionation. Care must be exercised, however, in the use of anti- θ serum to obtain the exact percentage of T cells. Greaves and Raff (20) have reported that different preparations of anti- θ serum vary considerably in their cytotoxicity for rosette-forming T cells. These authors present evidence that AKR autoantibodies are required in addition to anti- θ antibodies in order to kill some antigen-binding T cells, presumably because of the low density of θ -antigens on these cells. The two anti- θ sera used in our studies had the same cytotoxic titer against thymocytes, but the antiserum against C57BL thymocytes consistently killed 5-10% more antigen-binding cells than did the anti-C3H thymocyte serum.

In studies from different laboratories the percentages of T and B antigen-binding cells found in immunized and unimmunized mice varied considerably. Some workers have noted a marked increase in the percentage of T cells after immunization (18, 21), while others have not (17, 20). Under the conditions of our experiments, a significant change in the proportion of T and B cells could not be discerned after immunization. The variation in results is probably due to differences in the properties of the antigens, the dose and timing of the immunization, and to differences in the anti- θ sera.

The percentage of B cells among fiber-binding cells was

TABLE 4. Cytotoxic effect of anti- θ and anti-immunoglobulin on spleen fiber-binding cells of different specificities

Antigen on fiber*	Immunization	FBC†	% Killed	
			by anti- θ	by anti-immunoglobulin
Hemocyanin	—	145	30	61
BSA	—	110	34	62
BSA	BSA	225	29	57
Dnp-BSA	—	340	41	62
Dnp-BSA	Dnp-hemocyanin	842	39	56
<i>p</i> -Aminobenzoate-azo-BSA	—	159	33	52
Arsanilate-azo-BSA	—	100	34	56
Sulfanilate-azo-BSA	—	124	32	63
<i>p</i> -Toluenesulfonyl-BSA	—	212	43	54

* Fibers were derivatized with hapten-BSA conjugates at 0.25 mg/ml, hemocyanin at 2.5 mg/ml, and BSA at 5 mg/ml.

† Number of cells bound to edges of a 2.5-cm fiber segment.

determined by two independent assays, the binding of anti-immunoglobulin antibodies and the formation of EAC rosettes. Data from the two methods were in close agreement, and the sum of T and B cells accounted for virtually all of the fiber-binding cells (Table 3). This result also suggests that most or all of the T cells bound to the fibers were killed by the anti- θ sera.

The viability of cells bound to antigen-derivatized fibers exceeded 95%, even though the unfractionated cells included up to 35% dead cells. Although the reason for the failure to bind nonviable cells is not known, this property of fiber-fractionated cells facilitated subsequent assays by eliminating the background of dead cells. In all other respects, the results obtained in cytotoxicity assays with fiber-bound cells and with cells removed from fibers were equivalent to those obtained by conventional assays with unfractionated cell suspensions.

Attempts to determine whether T cells have immunoglobulins on their surfaces by use of anti-mouse immunoglobulin sera have not given uniform results (22). Moreover, the nature of the T-cell receptor for antigen has not been defined. We have found that up to 95% of the binding of cells to antigen-derivatized fibers can be inhibited by anti-mouse immunoglobulin. This finding does not necessarily indicate that the T cells are bound by immunoglobulin receptors. Steric hindrance, the presence of cytophilic antibodies, or several other factors (22) remain to be ruled out.

TABLE 5. Fluorescent staining of fiber-fractionated Balb/c spleen cells with anti- θ and anti-immunoglobulin*

Staining	Immunized (%)	Unimmunized (%)	Unfractionated
Anti-immunoglobulin	72†	69	45
Anti- θ	21	20	35

* Cells were fractionated on Dnp-BSA fibers. Dnp-hemocyanin was used as the immunogen.

† Expressed as % of nucleated cells stained.

It is an open question whether T- and B-cell receptors display the same range of antigen-binding specificities. Some studies suggest that T cells can recognize different antigenic structures than do B cells (23), although frequently the distinction between antigen-binding cells and cell stimulation has not been made. In our experiments, the proportion of T and B cells in populations fractionated with respect to several hapten or protein antigens was essentially the same, and comparable to values obtained with unfractionated spleen cell populations. Our findings suggest that under the conditions used, mouse T- and B-cell populations do not differ greatly in their *antigen-binding* specificities, at least for several protein and hapten antigens (Table 4). Various other antigens, including carbohydrate and cell-surface antigens, will have to be examined before this conclusion can be considered general.

T cells consistently showed a higher avidity than did B cells for the multivalent Dnp₁₀-BSA. For the monovalent ϵ -Dnp-lysine hapten, the affinities of T and B cells were found within experimental error to be the same. Möller, Bullock, and Mäkelä (manuscript in preparation) have reported that the affinity of B cells for a monovalent antigen is greater than that of T cells, and that this difference increased with time after immunization. Our experiments with monovalent antigens are probably consistent with these results, since the difference in affinity observed by these authors during the first week after secondary immunization was very small. It is clear that independent measures of affinity and avidity for each antigen are necessary in order to compare the relative range of specificities of T and B cells.

One hypothesis that is consistent with our findings with monovalent and multivalent Dnp antigens is that the receptors on T-cell surfaces may be arranged in clusters. The differences in the responses of T and B cells to antigens may not be a reflection only of the affinity of the individual receptors, but rather of the avidity and triggering threshold characteristic of the cell as a whole. No independent evidence exists as yet for clustering of T cell receptors before antigen binding. Although the clustering of intramembranous particles in the surface membranes of T cells has been observed by freeze-fracture techniques (24), the relationship of these particles to T-cell receptors remains to be defined.

The isolation of T or B cells of a single antigenic specificity makes possible a more precise analysis of their biochemical properties and biological functions. Preliminary results on the transfer of the fractionated cells into x-irradiated mice indicate that they include viable precursors of antibody-secreting cells.

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