ANTIFLUORESCEIN AFFINITY COLUMNS

Isolation and Immunocompetence of Lymphocytes That Bind Fluoresceinated Antigens In Vivo or In Vitro*

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Since 1969, when Wigzell and Andersson first described the removal of antigen-reactive lymphocytes with antigen-coated glass bead columns (1), numerous techniques have been reported for the elimination or purification of specific lymphocytes or lymphocyte subpopulations. These include, for example, the use of antigen or antibody bound to nylon fibers (2, 3), plastic tubes (4), plastic, polyacrylamide, or agarose beads (5–7), gelatin layers (8, 9), as well as "insoluble" antigen in the form of cellular monolayers of tumor (10), fibroblastoid (11), or red blood cell (RBC) (12) origin, rosetting (13), and, most recently, the fluorescence-activated cell sorter (FACS)[1] (14). All of these techniques are dependent on the binding of antigen via specific membrane receptors on normal or sensitized lymphocytes or antibody-forming cells (AFC). Elution of specific cells from insoluble matrices has been accomplished with variable yields by either nonspecific (agitation, shear) (1, 2) or specific (antigen) methods (4). With the exception of the FACS, each of these techniques requires the preparation of specific immunoabsorbents for each antigen used. Isolation with the FACS has the advantage of separation of virtually pure lymphoid cells which have been labeled with a fluorescent antigen (or antibody). We recently reported that cells that bind fluoresceinated antigen (FLAG) (or tolerogen) in vivo were isolatable with the FACS (15) and described the parameters for the detection of fate of these cells in tolerance vs. an immune response (16).

To isolate these antigen-binding cells in large quantities, however, we have developed a technique that takes advantage of fluorescent-antigen labeling as well as affinity chromatography over an insoluble matrix (i.e., antifluorescein-coupled beads). This technique is rapid and can be applied to any antigenic system into which fluorescein (FL) can be introduced. Elution is accomplished with fluoresceinated heterologous antigens. In this paper, we describe the principle of this technique and its application for the isolation of cells that have bound fluorescent antigens (T dependent and independent) in vivo and in vitro. Evidence for the immunocompetence of the isolated cells is presented.

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[1] *Abbreviations used in this paper:* ABC, antigen-binding cells; AFC, antibody-forming cells; AHF, aminoheptyl Ficoll; α-FL, antifluorescein; BSA, bovine serum albumin; E, eluted cell population; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FL, fluorescein hapten; FLAG, fluorescent antigen; KLH, keyhole limpet hemocyanin; MEM, minimal essential medium; O, original (unseparated) cell population; P, passed cell population; POL, polymerized flagellin; SGG, sheep gamma globulin; TNP, trinitrophenyl.

Materials and Methods

Animals. Male animals of the following strains were used at 2–6 mo of age: CBA/J (The Jackson Laboratory, Bar Harbor, Maine), C57BL/6 and C3H/St (West Seneca Breeding Labs, Buffalo, N. Y.), CBA/CaJ mice (University of Alberta Farms, Ellerslee, Alta.), and Lewis rats (Microbiological Associates, Bethesda, Md.).

Antigens and Fluorescein Conjugation. Polymerized flagellin (POL) from *Salmonella adelaide* SW1338 was the generous gift of Doctors C. Shiozawa and E. Diener of the University of Alberta. Trinitrophenylated sheep gamma globulin (TNP_8SGG) was prepared as described earlier (16). Aminoheptyl Ficoll (AHF) was prepared by activating Ficoll (mol wt, 400,000; Pharmacia Fine Chemicals, Piscataway, N. J.) with limiting amounts of cyanogen bromide and then adding a molar excess of diaminoheptane. The AHF was dialyzed extensively and conjugated at room temperature with trinitrobenzene sulfonic acid in cacodylate buffer (17) to yield $TNP_{20}AHF$. Keyhole limpet hemocyanin (KLH) was the kind gift of Dr. M. Rittenberg, University of Oregon Medical School.

Fluorescein isothiocyanate (FITC) conjugation (1 mg FITC/40 mg protein) was carried out at pH 9.5 in 0.05 M sodium carbonate at 0°C as modified from Goldman's procedures (16, 18). The final FL-labeled proteins and their average molar ratios of FL were: FL_5KLH (per 100,000 daltons), FL_5bovine serum albumin, FL_{15}horse gamma globulin, $FL_{3-5}SGG$, and $TNP_8FL_{2.5}SGG$. FL-POL contained 1 FL group per 40,000 dalton monomer. FL cells were prepared by adding 0.5 ml FITC (2 mg/ml) in carbonate to each 1 ml of cells (15% RBC or 10^8 nucleated spleen cells) for 20 min at room temperature and washing extensively. The number of FL groups on conjugated red cell or spleen cell membranes was not quantitatively ascertained, although all these cells were observed to be brightly fluorescent in a Leitz Ortholux II microscope.

Preparation of Antifluorescein [α-FL] and α-FL Columns. α-FL-KLH was produced by repeated injection of a male pony with 1 mg FL_5KLH in complete Freund's adjuvant. The α-FL was precipitated from the serum with sodium sulfate and then affinity purified by passage through and elution from a FL_{15}horse gamma globulin-Sepharose 4B column, which was prepared according to Schlossman and Hudson (7). This affinity purified α-FL was then conjugated to Sephadex G-200 or Sepharose 4B as above (7). Preliminary experiments determined that 3 ml of the α-FL beads had the capacity to specifically retain at least 10^8 FL-RBC, 25×10^6 FL lymphocytes, and 10^{-8} mol of FL in the form of FL-AHF (see below).

Column Isolation and Elution. Columns were prepared from disposable 10-ml syringes ("Plastipak," Becton-Dickinson & Co., Rutherford, N. J.) as follows. The rubber tip of the plunger was removed and cut transversely to yield an O ring which was then placed back in the barrel over fine nylon mesh to act as a support for the beads. Each column was then packed with 1 ml of Sephadex G-25 as a bottom layer and then 3 ml of the α-FL beads (Fig. 1). Preliminary experiments established that the G-25 layer greatly increased the flow rate of the columns. All beads were stored in phosphate-buffered saline (PBS) plus 0.1% sodium azide which was washed out with sterile PBS after the columns were poured. Routinely, columns were prepared in a laminar flow hood, washed with sterile PBS, and then exposed to 2,000 R γ-irradiation from a ^{137}Cs source. After irradiation, the columns were equilibrated with cold medium (except where indicated), the cells to be separated were carefully added (at $1-2 \times 10^8$/ml), and the columns clamped off for 5 min at room temperature. The columns were opened and washed with 15–50 ml of medium to yield a "passed" (P) cell fraction. At this point, 5–15 mg FL_5BSA at 5 mg/ml was added, the beads gently mixed, and the columns washed with 10 ml FL_5BSA at 500 μg/ml, followed by medium. This fraction is referred to as the eluted (E) cells. In preliminary experiments, equivalent amounts of FL_5BSA were added to the original (unseparated) and P cell preparations to control for the effect of excess FL_5BSA on the responsiveness of E cells. No effect of FL_5BSA on the responsiveness of any fractions was observed. It should be noted for the TNP experiments that FL and TNP do not cross-react as measured directly by plaque assay, inhibition of PFC, or cross-tolerance (D. Scott, unpublished observations).

All cell fractions were then washed three times in the appropriate medium and the cells examined for fluorescence and immunocompetence vs. specific and unrelated antigens. Since the number of E cells was quite small and may be deficient in accessory cell function, the competence of this fraction was tested by adding these cells to an aliquot of P cells (P plus E, reconstituted fractions), except where noted. The preparation of fluorescent antigen-labeled cells for each test situation is described in the Results section.

ISOLATION OF FLUORESCENT CELLS

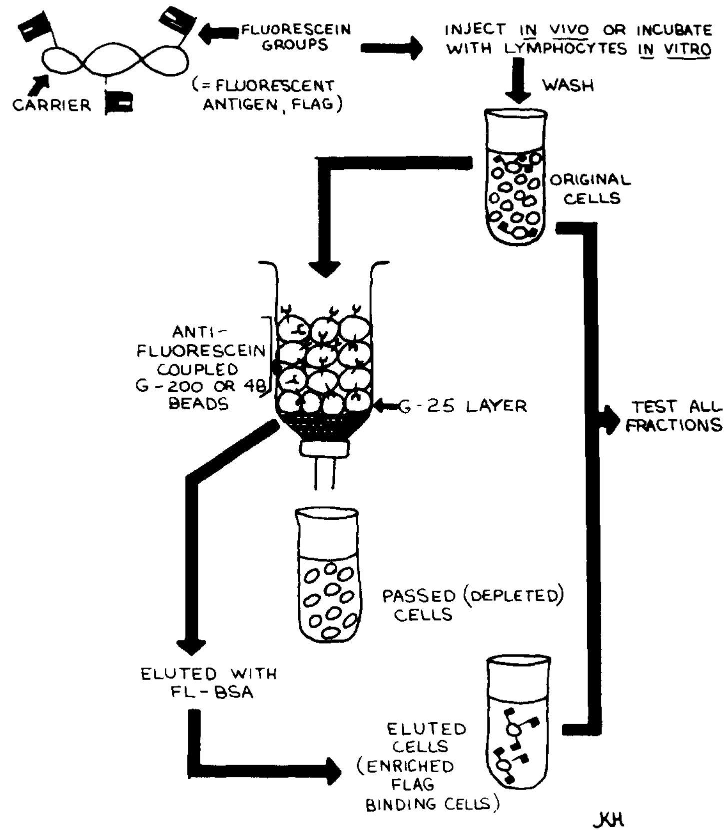

FIG. 1. Protocol for the isolation of fluorescein-labeled cells. See Materials and Methods for details.

Cell Culture Technique. In vitro responses were performed in modified Marbrook vessels (1.0–1.5 cm inner chamber diameter) with 10-15 × 10^6 mouse spleen cells in 1 ml minimal essential medium (MEM) plus 10% fetal calf serum (FCS), nonessential amino acids, 1 mM pyruvate, 1 mM glutamine, pen-strep (100 U and 100 μg/ml, respectively), and 5 × 10^{-5} M mercaptoethanol, except where noted. The outer chambers contained 10 ml of the above medium with 5% FCS, except for the POL experiments in which 50 ml was used with no mercaptoethanol. The antigens employed (and challenged doses per culture) were SRBC (4 × 10^6), TNP-SRBC (17) (4 × 10^6), TNP-Ficoll (50 ng), POL (100 ng), irradiated allogeneic and TNP-modified syngeneic spleen cells (responder:stimulator cell ratios = 10:1). All AFC responses were assayed after 4 days in Cunningham chambers (19) using SRBC or TNP-horse RBC (17) as targets. AFC to POL were measured as described by Diener (20). Cell-mediated lysis, on day 5 of culture, was performed at "killer":target ratios of 10:1–50:1 in 12 × 75-mm centrifuge tubes. Mixtures in a total vol of 1 ml were centrifuged at 800 rpm for 5 min at room temperature at the initiation of assay. ^{51}Cr release was measured after 4 h (except as noted) by the following formula:

$$\text{percent specific release} = \frac{\text{experimental cpm} - \text{medium background}}{\text{total (HCl) releasable cpm}} \times 100\%$$

Target cells included TNP-modified or unmodified C3H fibroblasts (clone 1D) or the C57BL tumor, EL4. These were generously provided by Dr. Linda Gooding.

Results

Basic Principles and Preliminary Experiments. In all experiments, lymphocytes were exposed to various FLAGs either by in vivo injection or by incubation in vitro (Fig. 1). Specific lymphocytes bind antigen via surface receptors and maintain antigen on their surface for at least minutes to hours or even days (16, 21, 22). Since the antigen is fluorescent, these cells can be directly observed (14, 15) and should theoretically bind to the α-FL beads. Furthermore, after unrelated (nonbound) cells are washed off, the specific cells should be competed off with excess FL-protein. This has been tested in several different systems.

Initially, columns were calibrated with FL-RBC as described in Materials and Methods. Each 3-ml column was found to retain 1–2 $\times$ 10^8 labeled RBC, up to 80% of which were elutable with FL-BSA. Preliminary experiments with spleen cells, labeled with FL-anti-immunoglobulin, established that up to 2.5 $\times$ 10^7 immunoglobulin-positive lymphocytes were bound by each column; however, less than 50% of these bound lymphocytes were elutable unless the beads were gently suspended with a Pasteur pipette when the FL-BSA was applied. The eluted cells, as expected, were >95% immunoglobulin positive.

To establish that antigen-binding cells (ABC) could be bound to and eluted from these columns, we examined the ABC from Lewis rats which had been injected with FL-SGG 1 h earlier (16). The washed but unseparated (original) spleen cell population contained 50 positive fluorescent lymphocytes per 100,000 cells. After passage through a 3 ml α-FL column, no positive cells were detectable in over 20,000 scanned (<5/100,000). The eluted cell fraction (approximately 1% of the total cell number applied) contained 30% positive cells (equivalent to 30,000 per 100,000) or a 600-fold purification.

However, these experiments merely demonstrated the capacity of our columns and the efficacy of elution of FL-labeled cells. These data say nothing about the immunocompetence of the various populations. The following experiments bear on this point.

Isolation of POL-Specific Lymphocytes. In this experiment, CBA/CaJ spleen cells were incubated with FL_1POL at 100 ng/ml or 10 μg/ml for 30 min at 37°C, washed three times, and then applied to the columns. During this time interval, antigen is bound to a similar portion of spleen cells at both concentrations of POL. It should be noted that 10 μg is normally a tolerogenic dose when cells are continuously exposed to POL for 6 h to 4 days; however, no reduction in responsiveness is observed if spleen cells are incubated with POL for only 30 min and then washed (reference 23 and results below). The immunocompetence of each fraction (or combined P plus E) was then determined, after further washing, by culturing the fractions with an immunogenic concentration of POL (100 ng/ml). As a specificity control, all fractions were cultured with sheep RBC. As can be seen in Table I, the P cells (at either dose of FL-POL) were significantly reduced in their responsiveness to POL (but not to SRBC). More importantly, the responsiveness of the P plus E fraction was restored to near normal levels. This indicates that the E fraction contains cells necessary for the AFC response to POL, whether immunogenic or tolerogenic levels of FL-POL were initially used to label.

Isolation of TNP-Specific Cells: Removal of AFC (B cell) but not Cytotoxic T-

TABLE I
*Isolation of AFC Precursors Specific for Polymerized Flagellin**

Group	Treatment group		AFC/culture (mean ± SE)	
			vs. POL	vs. SRBC
1	Tolerogenic dose‡ (10 μg/ml)	Original	2,613 ± 554	3,750 ± 782
2	"	Passed	407 ± 67	2,775 ± 460
3	"	Passed + eluted	1,560 ± 293	3,175 ± 805
4	Immunogenic dose (100 ng/ml)	Original	2,080 ± 342	4,500 ± 564
5	"	Passed	567 ± 81	3,550 ± 892
6	"	Passed + eluted	1,370 ± 360	2,900 ± 533

Group 2 significantly less than group 1 or 3 vs. POL and group 5 significantly less than group 4 or 6 vs. POL. No significant difference in SRBC responses.

* CBA/CaJ spleen cells were incubated for 30 min at 37°C with tolerogenic (10 μg/ml) or immunogenic (100 ng/ml) doses of fluoresceinated POL(FL_1POL), washed, and separated on anti-FL columns as described. All fractions were washed and challenged in Marbrook vessels with 100 ng POL and 0.02% SRBC. AFC were assayed on day 4. Note, no response to POL was seen if the cultures were not challenged with 100 ng POL. Thus the isolation procedure per se did not trigger these cells.

‡ See text.

Cell Precursors. To determine if TNP-reactive lymphocytes could be isolated with α-FL columns, C3H/St mice were injected intravenously with 1 mg of $TNP_8FL_{2.5}$SGG, and their spleen cells prepared 1 h later. Washed spleen cells were passed through the columns as described above and cultured as follows. One set of original unseparated (O), P, and P plus E fractions was cultured with 50 ng TNP_{20}AHF or SRBC. Another aliquot of O, P, and P plus E cells was cultured with 2,000 R γ-irradiated TNP-C3H splenocytes (or irradiated C57BL spleen cells as a specificity control). As can be seen in Table II, the AFC response to TNP_{20}AHF was significantly reduced in the P fraction, but was restored when E cells were added. The response to SRBC was the same in all cultures.

Interestingly, when fractions from the same pool of spleen cells were tested for the generation of cytotoxic T cells in response to TNP-modified syngeneic cells (24), no depletion of activity was observed in the P-cell fraction (Table III). In fact, in several experiments, the cytotoxic response of P or P plus E fractions was increased compared to the unseparated (O) cells. Further attempts to remove cytotoxic T-cell precursors, after brief incubation of responders with FL-conjugated stimulating spleen cells and column treatment, were also unsuccessful (data not shown).

Enrichment of Cytotoxic T Lymphocytes. The previous experiment demonstrated that TNP-specific AFC precursors were isolatable with these columns, whereas the precursors of TNP-specific cytotoxic T lymphocytes were not removed. This could be because hapten-reactive cytotoxic lymphocytes recognize the hapten and part of the *H-2* complex (24), and the binding to TNP on a heterologous carrier, if it occurs, was insufficient under our isolation conditions for cell separation. However, preliminary experiments, using fluoresceinated syngeneic or allogeneic cells, failed to remove cytotoxic T-cell precursors specific for these antigens (data not shown). Therefore, it became important to demon-

TABLE II
*Isolation of TNP-Specific AFC Precursors**

	Mean PFC ± SE/culture (day 5)	
	vs. TNP	vs. SRBC
Original	797 ± 73	767 ± 112
Passed	156 ± 45	713 ± 164
Passed + eluted	742 ± 64	927 ± 47

* C3H mice were injected i.v. with 1 mg $TNP_8FL_{2.5}$ SGG (−1 h), their spleens washed and separated on α-FL columns as described. All fractions were cultured in Marbrook vessels with TNP-Ficoll (50 ng/culture), SRBC (0.02%/culture), or no Ag (background). Background PFC to TNP (~200) and SRBC (~80) have been subtracted.

TABLE III
*Attempted Isolation of Killer Cell Precursors**

Killers		Percent ^{51}Cr release‡		
	Targets:	TNP-CL1D	CL1D	EL4
Original		21.1 ± 2.1	−1.2 ± 1.9	64.2 ± 2.7
Passed		37.1 ± 4.8	−0.4 ± 1.8	67.1 ± 1.1
Passed + eluted		47.8 ± 1.9	−0.4 ± 2.5	73.6 ± 0.7

* C3H mice were injected i.v. with 1 mg $TNP_8FL_{2.5}$SGG(−1 h), their spleens washed and separated on α-FL columns as described. All fractions were cultured in Marbrook vessels with TNP-modified C3H spleen cells (responder:stimulator ratio = 10:1) and tested against TNP-modified and unmodified C3H cells (CL1D). Aliquots of each fraction were also sensitized separately vs. C57BL spleen cells and tested against EL4. ^{51}Cr release assay on day 5 at 30:1 killer to target ratio (4-h assay).

‡ Percent ^{51}Cr release = (experimental cpm − medium background cpm)/(total releasable cpm (HCl)), where background = 14.5% (TNP-CL1D), 15.3% (CL1D), and 11.4% (EL4) and total releasable = 76.1% (TNP-CL1D), 77.7% (CL1D), and 82.3% (EL4).

strate whether any T-cell population was isolatable using α-FL columns. To test this, we employed BALB/c anti-EL4 (H-2^d anti-H-2^b) cytotoxic peritoneal exudate lymphocytes, which are a potent population of killer cells (25) and have been shown to bind to EL4 cells or cell monolayers in vitro (9, 26). BALB/c peritoneal cells, harvested on day 11 after an intraperitoneal injection of 25 × 10^6 EL4 leukemia cells, were mixed at a 20:1 (BALB:EL4) ratio with FL-EL4 and centrifuged for 5 min at 800 rpm to promote cell contact. The cell pellets were gently resuspended and briefly warmed to room temperature, after which an aliquot was removed (original) and the remainder passed through an α-FL column. The original, passed, and FL-BSA eluted cells were washed and tested in a ^{51}Cr release assay (2 h) vs. EL4 targets. Aliquots of O, P, and E cells were also checked for the frequency of FL-EL4 and FL-EL4:BALB lymphocyte "rosettes" since 100% of the added EL4 are fluorescent and easily identifiable in a ultraviolet microscope. As a control, immune BALB/c peritoneal cells were mixed with sham-labeled nonfluorescent EL4 and "separated" over identical

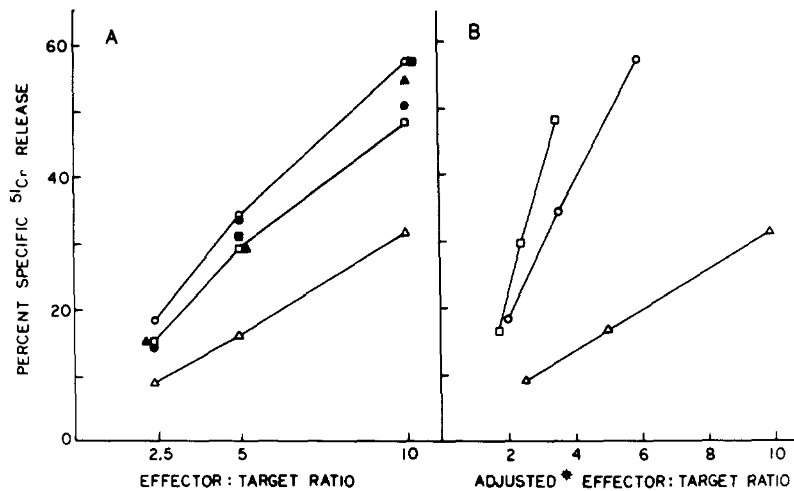

FIG. 2. Depletion and enrichment of killer cell activity. BALB/c anti-EL4 peritoneal exudate cells were centrifuged with FL-EL4 and then passed through an α-FL column as described in the text to yield passed (△) and eluted (□) populations. These fractions and the original, unseparated population (○) were then tested against ^{51}Cr-labeled EL4 in a 2 h chromium release assay at various killer:target cell ratios. As a control, BALB/c anti-EL4 were also centrifuged with sham-labeled EL4 and "separated" on the columns (▲, ■, ●). In Fig. 2 A, the effector:target ratio was not adjusted for the FL-EL4 which were present in each fraction. In Fig. 2 B, the number of FL-EL4 in each fraction was subtracted to yield an adjusted effector:target cell ratio, assuming that FL-EL4 acted as "cold" competing targets.

columns. The results (Fig. 2) show that passage over α-FL columns significantly depleted the cytotoxic activity of BALB/c cells previously bound to FL-EL4, whereas cytotoxic BALB/c cells exposed to sham-labeled EL4 were present in all column fractions, i.e., were not separated. The results are even more striking when one takes into consideration the FL-EL4 also present in the eluted cell population, since these FL-EL4 are not ^{51}Cr labeled and would be expected to "compete" with ^{51}Cr-EL4 for effector cell contact (Fig. 2 B). Note, however, that the degree of competition by FL-EL4 was not directly tested, so the results in Fig. 2 A represent an underestimate of killer cell activity in the eluted fraction, while Fig. 2 B is probably an overestimate of this activity. Nevertheless, these data indicate that cytotoxic T cells can be isolated by procedures similar or identical to those used for the isolation of AFC precursors (B cells).

Discussion

The present experiments indicate that α-FL columns can be used to isolate antigen-reactive lymphocytes specific for a variety of antigens, if these antigens are fluoresceinated (FLAGs) and bound to specific lymphocytes in vitro and in vivo. The method is rapid, versatile, and requires only one immunoabsorbent, the α-FL beads. So far, we have used this methodology to isolate AFC precursor (B) lymphocytes specific for POL and TNP (Tables I and II), plaque-forming cells specific for SRBC and TNP (L. Greeley and D. Scott, unpublished observations), as well as alloantigen-specific cytotoxic T lymphocytes (Fig. 2). Further purifica-

tion of cytotoxic T cells is currently being achieved with EDTA, which is known to separate killer cells from their targets[2] (10).

Although cytotoxic T lymphocytes have been separated, we have not been able to consistently isolate other T-cell populations, such as helper T cells, cells that proliferate in vitro in response to soluble antigen, or the precursors for cytotoxic T cells (unpublished data). Previous workers also failed to remove various T-lymphocyte populations with antigen-affinity columns (1, 6). It is noteworthy that Rubin recently reported (27) that affinity columns may avidly retain a suppressor T-cell population; thus, the passed cell fractions in his experiments gave augmented helper reactivity, but diminished B-cell activity. Our columns may also retain a suppressor population as evidenced by the increased cytotoxic response of the passed cells in Table III. However, this has not been rigorously tested.

It should be noted that the α-FL columns do not yield "pure" populations at present, as can be achieved with the FACS, although these columns can be used as a substitute "poor man's cell sorter." However, this methodology is more rapid than the FACS, yields immunocompetent populations, and can readily be carried out under aseptic conditions. Further purification of the eluted cells can presumably be achieved by more extensive washing of the columns before elution or by rerunning the eluted cells.

Present methods for the isolation of specific lymphocytes require their interaction, via their receptors, with antigen or anti-idiotype serum (28). This interaction, by definition, means that they are not longer technically immunologic virgins (although their membranes may still be "intact"). It is clear from the experiments with FL-POL, with tolerogenic or immunogenic concentrations of antigen, that these cells are still immunocompetent. That is, the brief interaction with antigen, per se, can be done under conditions that do not significantly induce nor suppress a subsequent response. Thus, purified antigen-reactive cells could be isolated at various stages of the immune response or tolerance induction and studied in large numbers. In addition, purified antigen-specific cells may now be cloned, after viral transformation or cell fusion, and analyzed in much the same way as myeloma cells were previously characterized.

Summary

A new method for the isolation of specific immunocompetent lymphocytes has been described in which lymphocyte populations are exposed to fluoresceinated antigens (FLAGs) in vivo or in vitro, and the FLAG-binding cells retained on antifluorescein affinity columns. Specific cells are then eluted with an unrelated FL-labeled protein and shown to be fully immunocompetent. This methodology has been applied successfully in diverse antigenic systems including polymerized flagellin and TNP-specific B cells and alloantigen-reactive cytotoxic T lymphocytes. The method is rapid, inexpensive (requiring only antifluorescein beads), and can be applied to any antigens (or antibodies) in which a fluorescein group can be introduced.

[2] Singer, K. Hiemstra, C. Johnston, D. B. Amos, and D. W. Scott. Cytotoxic activity of affinity column-purified alloantigen-specific T lymphocytes. Manuscript in preparation.

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