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M.H. Julius[+], Elizabeth Simpson[o] and L.A. Herzenberg

Department of Genetics, Stanford University School of Medicine, Stanford

A rapid method for the isolation of functional thymus-derived murine lymphocytes*

A rapid method is described for effectively removing immunoglobulin-bearing cells from either primed or unprimed mouse spleen and lymph node cell suspensions. Incubation of cell suspensions in nylon wool columns for 45 min at 37 °C resulted in a 9 to 100-fold depletion of immunoglobulin-bearing cells and a complementary 1.5 to 2-fold enrichment of T cells in the column effluent populations.

The effluent population, derived from passage of spleen cells through these columns, was virtually devoid of B precursor and memory cell activity, but contained all of the helper cell and cytotoxic effector cell precursor activity when compared to unfractionated spleen cells.

1. Introduction

The ability to easily prepare highly enriched, unselected and unaltered populations of thymus-derived (T) lymphocytes will aid in studies of their role in immune systems. The reported methods either use "naturally" enriched T cell sources such as thoracic duct lymphocytes, obtained under technically demanding conditions, or *in vitro* enrichment.

In vitro methods for enriching T lymphocytes from spleen or lymph node populations can be generally classified into two categories. The first involves the specific elimination of B lymphocytes based on presence of known membrane components. B cells, which carry relatively large amounts of membrane-bound immunoglobulin (Ig) [1–3], may be selectively killed by treatment with anti-light chain antibody (anti-kappa) plus complement [4], or may be depleted by passage through a column of anti-immunoglobulin antibody coated on plastic beads [5]. However, the requirement for large quantities of anti-immunoglobulin sera and/or lengthy column preparation time limits the ease with which these methods can be used preparatively. Moreover, whether important but subtle physiological or chemical changes of membrane structures in purified T cells result from incubation in complement, antiserum or from immunoglobulin interaction on the beads is not known.

Another method utilizing differential membrane components (which suffers from similar drawbacks) was described by Basten et al. [6]. B lymphocytes have receptors for a structural component located on the Fc fragment of certain classes of Ig molecules. The affinity of the receptors appears to be greatly increased when the Ig are complexed with antigen. Therefore, B cells may be depleted from lymphocyte populations by pretreatment of the cells with anti-fowl gamma globulins (FGG) and passage through a plastic bead column coated with FGG. The anti-FGG, which appears to be loosely bound to the B cells, complexes with the FGG on the column and this apparently binds the B cells tightly to the column.

The second category of methods described for depleting B cells from lymphoid populations consists of essentially empirically derived procedures. For unknown reasons, B lymphocytes tend to stick preferentially to glass beads [7, 8, P.B. Adams, unpublished data], nylon wool [9] or cotton wool [10]; however, multiple passages through columns of either of these materials, resulting in considerable non-specific cell losses, are required to bring about effective depletion. In none of these methods is greater than 90 % depletion of B cells with greater than 50 % recovery of T cells observed. The low recoveries may be due to selective losses of T cell subsets.

This publication reports a simple and reproducible method for efficiently eliminating Ig-bearing cells from murine spleen and lymph node cell suspensions with excellent T cell recoveries. Dr. H.W. Kreth, working in this laboratory, found that passage of human peripheral blood lymphocytes through long nylon fiber columns at 37 °C [11] resulted in a 2-3-fold depletion of Ig-bearing cells. Using a more rapidly carried out modification of this method, we have been able to recover 50 % to 90 % of the T cells in mouse spleen and lymph node suspensions with a concomitant 10 to 100-fold depletion of B cells. One absorption in a nylon wool column yields a population of spleen cells containing 85 to 90 % T cells, less than 5 % Ig-bearing cells, and approximately 10 % of cells not identifiable as either. Similar processing of lymph node cells yields a population containing > 95 % T cells and less than 1 % Ig-bearing cells.

2. Materials and methods

2.1. Mice

Male and female mice of the congenic strain BALB/cN (Ig^a) and BAB/14 (Ig^b) were used at the age of 2 to 6 months. (BAB/13 were kindly supplied by Dr. M. Potter, and BAB/14 were derived in this laboratory by one further backcross to BALB/cN and then inbreeding the resulting heterozygotes.)

2.2. Adoptive transfers and antibody assays

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+ Becton-Dickinson Fellow, Department of Genetics, Stanford University.

o Visiting Associate, Laboratory of Immunology, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20015, USA.

Correspondence: Michael H. Julius, Department of Genetics, Stanford University School of Medicine, Stanford, Calif. 94305, USA.

Abbreviations: DNP: 2,4-Dinitropnenyl **TNP:** 2,4,6-Trinitrophenyl **KLH:** Keyhole limpet hemocyanin **PFC:** Plaque-forming cells **PBS:** Phosphate buffered saline **B cell:** Bone marrow-derived cells **T cell:** Thymus-derived cell **RAMIG:** Rabbit anti-mouse Ig **RAMT:** Rabbit

2.3. Nylon wool columns

The sterile nylon wool in LP-1 Leuko-Pak Leukocyte Filters (Fenwal Laboratories, Morton Grove, Illinois) was used without any further preparation for experiments involving *in vivo* transfer of cells after column passages However, for experiments in which the nylon column effluent cells were immunized *in vitro* (see section 2.8) the nylon wool had to be washed to remove a toxic product which otherwise decreased *in vitro* survival of these cells. The washing procedure was carried out by soaking the contents of up to 3 Fenwal filter packs in a one liter beaker (presoaked in normal saline for 2 h at 37 °C and rinsed 3 times in glass-distilled water at room temperature) containing double glass-distilled water at 37 °C. Three or four changes of water were made over a period of 1 week, and the nylon then wrung out and dried in a 37 °C incubator for 2–3 days. About 0.6 gram aliquots of washed and dried nylon wool were then packed into the barrel of 12 ml plastic syringes (Monoject, Sherwood Medical Industries, Inc. St. Louis, Mo.) up to the 6 ml mark and the packed syringe barrels replaced in the plastic syringe covers for autoclaving. Subsequently, the sterile nylon columns were rinsed with about 20 ml of Dulbecco's phosphate buffered saline [14] containing 5 % heat-inactivated fetal calf serum. (This medium was used in all column experiments and subsequent staining reactions.) The columns were drained of excess medium and then replaced in the sterile syringe covers, the lids sealed with masking tape to prevent evaporation, and placed in an incubator at 37 °C at least 1 h before loading of cells.

Cell suspensions, prior to loading onto the nylon wool columns, were filtered through glass wool at 22–25 °C to remove dead cells and most macrophages. A total of 1 x 10^8 to 1.5 x 10^8 cells, in a volume of 2 ml, were loaded onto the column (which remains in the vertical position throughout the experiment) and subsequently washed into the nylon wool with 0.5–1 ml of warm (37 °C) medium. The columns were replaced in the sterile syringe covers and the lids resealed and left for 45 min at 37 °C.

The columns were then washed slowly (so as not to generate a fluid head) with warm (37 °C) medium and the first 25 ml of effluent was collected in 15 ml conical tubes and the cells pelleted at 290 x g for 10 min in the cold (4 °C) (these centrifugation conditions were used throughout these and all staining experiments). Viable cells excluding trypan blue were counted in a hemocytometer.

The procedures used for experiments involving *in vivo* transfer following column separation were the same as the above, with the exclusion of the nylon wool pre-washing and autoclaving of the columns, and columns during the incubation periods were sealed with Parafilm.

2.4. Antisera

A rabbit antiserum prepared against a saline EDTA extract of mouse thymus (RAMT) and absorbed with mouse bone marrow was shown to be specific for T cells [15]. This serum had anti-BSA (bovine serum albumin) (BSA was present in the immunizing medium) and a trace of anti-IgG2a immunoglobulin activity as detected by radioimmune precipitation which were removed by passage through immunoadsorbent columns.

Rabbit anti-mouse Ig (RAMIG) was prepared by repeated injections of rabbits with ammonium sulfate purified mouse Ig in complete Freund's adjuvant. Ig fractions of this antiserum were prepared by ammonium sulfate precipitation.

Goat anti-rabbit Ig (GARIG) (a gift of Dr. L. Rosenberg) was made by repeated injections of goats with DEAE-cellulose purified rabbit Ig in complete Freund's adjuvant. Rhodamine conjugates of the ammonium sulfate purified Ig of this antiserum were prepared according to the method of Cebra and Goldstein [16] and those with a rhodamine/protein molar ratio of about 3 were used in staining experiments.

2.5. Indirect immunofluorescence staining for T and Ig-bearing cells

Pelleted cells (1 x 10^7 – 2 x 10^7) were resuspended in a first layer (0.25 ml) of either RAMT at 0.5 mg/ml or RAMIG at 1.2 mg/ml and left at 0° – 4 °C for 20 min. The cell suspension was then pelleted through neat heat-inactivated fetal calf serum and subsequently washed in 2 ml of medium. This pellet was resuspended in the second layer (0.25 ml) of rhodamine-conjugated GARIG at 0.2 mg/ml and left at 0° – 4 °C for 20 min. The cells were washed as described above and the final pellet resuspended in 0.05 ml neat fetal calf serum. Smears were prepared and fixed in 95 % ethanol for 15 min and subsequently mounted in 9 : 1 glycerol-phosphate buffered saline.

2.6. Fluorescence microscopy

A Zeiss microscope with an HBO 200 mercury arc (OSRAM) light source was used. Cells were examined with darkfield illumination using a Zeiss 1.2/1.4 NA oil immersion darkfield condensor and a Zeiss 100 x oil immersion planachromat objective with an iris diaphragm. Combination of a Zeiss PIL 546 nm excitation filter and two layers of Kodak Wratten No. 23A gelatin barrier filter were used to detect fluorescence.

Cells were first examined under white light darkfield conditions and those with intact plasma membranes which under fluorescence illuminating conditions exhibited speckled, ringed or crescent fluorescence were considered positive.

2.7. Plaquing assay

A modified version of the hemolytic plaque technique was used [17]. Trinitrophenyl-coated sheep erythrocytes [18] were used to assay anti-DNP PFC. Guinea pig serum at a final concentration of 1/24 was used as a complement source.

2.8. *In vitro* sensitization and cytotoxic assay

The *in vitro* sensitization method of Wunderlich and Canty [19] was used to sensitize normal BALB/cN spleen cells, which had either been passed through glass wool alone, or both glass wool and nylon wool columns, to C57BL/6N antigens. Because macrophages essential for the induction of T cell cytotoxicity [20] had been removed by the glass wool columns, it was necessary to add back small numbers of macrophages prior to sensitization. 2 x 10^6 BALB/cN peritoneal wash cells were added to each aliquot of 20 x 10^6 cells to be sensitized and these were incubated in 35 x 10 mm petri dishes (Falcon Plastics 3005, Los Angeles, Calif.) together with 1 x 10^6 2500 rad irradiated C57BL/6N cells for 5 days on a rocking platform in a CO_2 incubator. To control for any activity of the peritoneal wash cells alone, 2 x 10^6 were mixed with 20 x 10^6 (BALB/cN x C57BL/6N)F_1 spleen cells and incubated with antigen as above. Cells for sensitiza-

tion were suspended in Eagle's fortified medium and fed daily with nutrient medium [21].

After 5 days sensitization the cells were harvested and their cytotoxicity measured according to the method of Canty and Wunderlich [22]. Briefly, they were suspended at 2 x 10^6, 1 x 10^6 and 0.5 x 10^6 viable cells per ml in Eagle's medium with 10 % fetal calf serum and mixed with 5 x 10^5 ^{51}Cr-labeled EL4 cells (C57BL/6N lymphoma) in 35 x 10 mm petri dishes in quadruplicate samples. They were incubated at 37 °C on a rocking platform for 4 h in a CO_2 incubator and the ^{51}Cr released into the supernatants counted. The percent cytotoxicity, relative to 100 % release from a freeze-thawed control, was calculated.

3. Results

3.1. Removal of immunoglobulin-bearing cells

Conditions were established where Ig-bearing cells from either normal or KLH-primed spleen or normal lymph node cell suspensions could be separated from the remaining lymphocyte population. This was accomplished by incubating the cells in a nylon wool column for 45 min at 37 °C (see section 2.3) and then washing the nonadherent cells through with additional buffer. Aliquots of cells before and after nylon wool incubation were indirectly stained with either RAMIG as a marker for immunoglobulin-bearing B cells or RAMT as a marker for T cells.

Table 1 illustrates that spleen cells contained about the same proportions (45 %) of T and B cells before nylon wool incubation. Column effluent cells showed a 9 to 20-fold depletion of Ig-bearing cells and a concommitant 2-fold enrichment of T cells. In fifteen experiments (some of which are shown in Table 1), the effluent populations contained between 84–91 % T cells and 2–5 % B cells. The recovery of T cells was between 50 and 89 %.

Lymph node cell populations contain about twice as many T (68–65 %) as B (31–34 %) lymphocytes (Table 1). In two experiments, column effluent cells showed a 50 to 100-fold depletion of Ig-bearing cells with a complementary 1.5-fold enrichment of T cells. The recovery of T cells in these experiments (50 % and 54 %) was similar to that of splenic T cell recovery. The reasons for the more efficient depletion of lymph node Ig-bearing cells on these columns is not clear.

The viability of column effluent cells was always > 90 % (as assessed by trypan dye exclusion), irrespective of the viability in the starting population. However, we have noted that low viability (< 80 %) in the starting population correlated with greater Ig-bearing cell contamination in the effluent population. Therefore, cell suspensions were always filtered through glass wool before loading onto the nylon wool columns. Both the initial cell concentration and the total number of cells loaded onto the column appear to affect the efficiency of the depletion of Ig-bearing cells. Therefore, in these experiments we have used up to 1.5 x 10^8 cells in 2 ml.

The contaminating splenic Ig-bearing cells found in the nylon wool effluent population, under efficient loading conditions, appear to be a subpopulation of the splenic lymphocytes which stain with the anti-Ig reagent. Experiment 4, Table 1 shows the effect of repassing the effluent population through nylon wool. The first pass (4,1) resulted in a 12-fold depletion of Ig-bearing cells and an 89 % recovery of viable T cells. One would expect then, that a second pass under the same conditions would result in a further 12-fold depletion of Ig-bearing cells assuming a random interaction of these cells with the nylon fibers. However, only a 2-fold (4,2) further depletion was accomplished, indicating that the Ig-bearing cells remaining after the first pass through nylon wool represent a less adherent population of cells. Further, most of the Ig-bearing cells in the effluent population after one pass through nylon wool, appear to have less membrane associated Ig determinants by immunofluorescent staining, *i.e.* the cells stain much more lightly. The few cells with average staining were removed by the second pass.

In the two experiments done with lymph node cells the contaminating Ig-bearing cells showed the same staining morphology and intensity as those prior to nylon wool passage.

Table 1. Recovery of "T cells" after nylon wool filtration

	% Stained cells[b]				% Recovery of viable cells	
	Before filtration		After filtration[c]		Total	T cells
	Ig	T	Ig	T		
		46	2	91		84
		43	4	84		85
		45	2	89		67
		43	4	84		89
		84	2	90		68
		46	5	87		54
		46	4	89		89
		58	0.4	97		80
			0.6	96		

a) Pools of 3–5 mice used for each experiment.
b) Indirect stain with either RAMIG or RAMT and rhodamine-conjugated GARIG.
c) Cells filtered through nylon wool columns at 37 °C as described in section 2.3.
d) Nylon column effluent cells from 4,1 repassed through a second column under the same conditions.
e) Primed with 100 μg KLH (aqueous) 3 months prior to sacrifice.
f) Cells from this experiment were used as a source of cooperators – see Table 2.

3.2. Removal of B precursor and memory cells

To test whether nylon wool incubation effectively removes functional B cells, long-term (2–3 months) KLH primed spleen cells were passed through a nylon wool column and the effluent population tested for both primary anti-DNP and secondary anti-KLH responses in an *in vivo* transfer system. Irradiated animals received varying numbers of KLH-primed nylon column effluent cells intravenously. Animals were challenged with 100 μg of alum-precipitated DNP-KLH intraperitoneally on day 0, boosted with 10 μg of aqueous DNP-KLH intravenously on day 5, bled and sacrificed on day 12. The proportion of Ig and T-bearing cells in the nylon wool effluent population used in this experiment is shown in Table 1, Exp. 6.

As illustrated in Table 2, 5 x 10^6 KLH-primed spleen cells (untreated) contained precursor cells for DNP and memory cells for KLH. Animals responded with 21 x 10^3 direct anti-TNP plaques per spleen and gave an anti-KLH hemagglutination titer of 1100. However, transfer of the same number of KLH-primed nylon column effluent cells resulted in a 10-fold depletion in the anti-TNP plaque-forming response (2 x 10^3 plaques/spleen) and close to 100-fold depletion in the anti-KLH hemagglutination titer. This demonstrates that the nylon column filtration had effectively removed not only the DNP precursor population, but also the KLH B memory cells, from the KLH primed spleen.

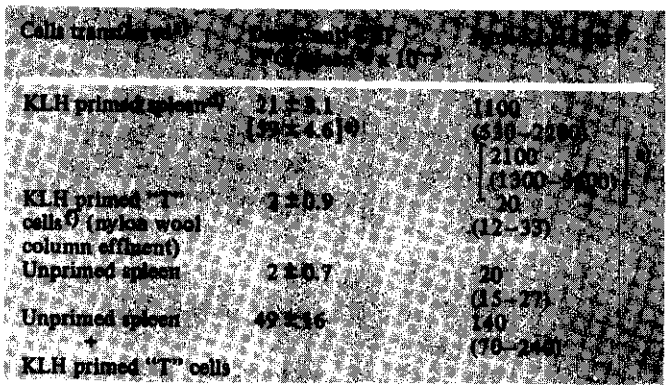
3.3. Recovery of helper cell function

The nylon column effluent population contained all the cooperating activity found in whole KLH-primed spleen when corrected for the proportion of T cells in the recovered population (89 %). To assay for cooperators, the same transfer system outlined in section 3.2. was used, but 5 x 10^6 unprimed spleen cells were given in addition as a source of DNP precursors. The data in Table 2 illustrate that when either 5 x 10^6 unprimed spleen or 5 x 10^6 KLH-primed nylon wool effluent cells were transferred alone, the anti-TNP response did not rise above background (2 x 10^3PFC/spleen). However, when 5 x 10^6 nylon wool column effluent cells were transferred in combination with 5 x 10^6 umprimed spleen, the animals responded with 49 x 10^3 PFC/spleen, indicating that the effluent population was able to supply KLH cooperators for the DNP precursors in the unprimed spleen.

Comparison of the cooperating activity of whole spleen with the activity of the nylon wool effluent shows that effluent cells are roughly twice as active. Transfer of 5 x 10^6 cells from whole KLH-primed spleen gave 21 x 10^3 PFC/spleen, whereas transfer of 5 x 10^6 KLH-primed nylon wool effluent cells with precursor cells gave 49 x 10^3 PFC/spleen (see Table 2 and Fig. 1). This is consistent with the roughly twofold increase in T cells observed in the nylon wool effluent population as compared to whole spleen.

The data in Fig. 1 confirm this conclusion. The cooperator activity of whole KLH-primed spleen, when corrected for T cell content, is essentially equal to the cooperator activity of the KLH-primed nylon wool effluent population similarly corrected. Thus, 10^7 whole KLH-primed spleen containing 4.5 x 10^6 T cells (as measured by immunofluorescence) gives 59 x 10^3 TNP PFC/spleen and 5 x 10^6 KLH-primed nylon wool effluent cells containing 4.5 x 10^6 T cells give 49 x 10^3 TNP PFC/spleen when combined with 5 x 10^6 unprimed spleen as a source of precursor cells. Similar results (not shown here) were obtained in another experiment.

Table 2. Removal of B precursor and memory cells by nylon wool



a) 600R irradiated animals received 5 x 10^6 of the noted cell type and 100 μg alum-precipitated DNP-KLH on day 0. Animals were boosted with 10 μg of aqueous DNP-KLH on day 5, bled and sacrificed on day 12. There were 4 animals/group, tested individually.
b) Arithmetic mean ± standard error.
c) Geometric mean. Numbers in parentheses represent the standard error about the geometric mean. Passive hemagglutination assay using KLH-conjugated sheep erythrocytes prepared by the method of Avrameas [23].
d) Animals were primed with 100 μg of aqueous KLH 2–3 months prior to sacrifice.
e) Numbers in square brackets are the anti-TNP PFC/spleen and anti-KLH titer resulting from the transfer of 1 x 10^7 KLH primed spleen cells.
f) KLH-primed spleen depleted of Ig-bearing cells by passage through nylon wool column. See Table 1, Experiment 6 for staining results.

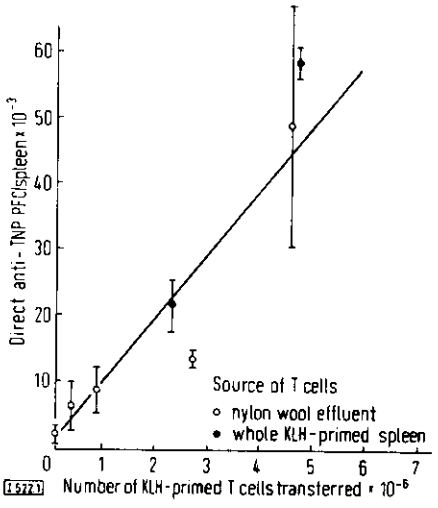


Figure 1. Cooperation between KLH-primed "T" cells and normal spleen cells in anti-TNP response to DNP-KLH. Irradiated animals (600 rad) received 5 x 10^6 unprimed spleen cells along with graded numbers of KLH-primed nylon wool effluent cells (○) or either 5 x 10^6 or 1 x 10^7 whole primed spleen (●) intravenously. They were given 100 μg of alum-precipitated DNP-KLH intraperitoneally on day 0 (with the cells), boosted with 10 μg of aqueous DNP-KLH intravenously on day 5, bled and sacrificed on day 12. There were 4 animals/group which were individually tested and each point represents the arithmetic mean of the anti-TNP PFC response per spleen in one group. The number of T cells transferred was assessed by immunofluorescent staining.

3.4. Recovery of cytotoxic precursor cell activity

Cytotoxic precursor cell activity, another well-characterized T cell function, is completely recovered in the nylon wool effluent population. Following five days incubation with C57BL/6N antigen, the cytotoxic activities of peritoneal wash

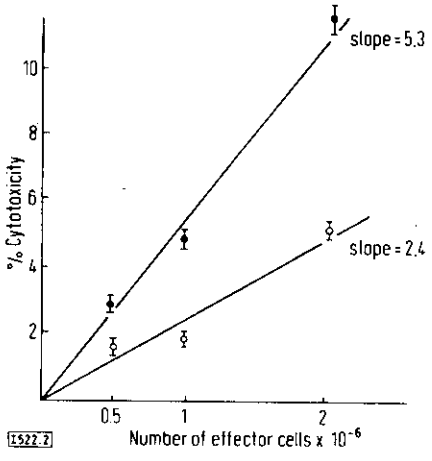


Figure 2. Cytotoxicity of nylon wool filtered and unfiltered spleen cells sensitized *in vitro*. Nylon column filtered (○) or unfiltered (●) BALB/cN spleen cells were sensitized *in vitro* for 4–5 days with 2500 rad irradiated C57BL/6 spleen cells. Various numbers of sensitized cells were then incubated with 5 x 10^6 ^{51}Cr-labeled EL4 cells for 4 h. ^{51}Cr released was measured and expressed as "% cytotoxicity" relative to 100 % release from a freeze-thawed preparation.

cells plus either normal spleen cells or nylon wool effluent cells were measured against ^{51}Cr-labeled EL4 (C57BL/6N lymphoma) cells. The values for percent of cytotoxicity at each attacker target cell ratio were plotted for each of the cell populations and the slopes of the lines compared (Fig. 2).

The results obtained with this system paralleled those in the cooperative system. The nylon wool effluent population had roughly twice as much precursor cell activity (5.3 % cytotoxicity/10^6 sensitized cells) as the original spleen (2.4 % cytotoxicity/10^6 sensitized cells) which is accounted for by the twofold enrichment of T cells in the effluent population. The activity of peritoneal wash cells incubated with F_1 spleen cells was negligable. Another experiment, not shown here, gave similar results.

It was found that neither the glass wool nor the nylon column effluent populations in these experiments could be sensitized without the addition of a source of macrophages, as these cells are efficiently removed by glass wool filtration. 1 x 10^6 perito-

a subpopulation of B cells not involved or less efficient in either precursor or memory cell function. An attempt to remove them from the effluent population by passing the latter through another nylon wool column did result in some further depletion, but mainly of the more brightly staining cells. We do not know what significance to attach to the dimly stained cells which are not depleted even by repassage through a second column.

Recovery of T cells from spleen and lymph node cell suspensions in the column effluents ranged from 50 % to 89 %. The T cell loss, at least with respect to the spleen cell experiments, is not selective for any possible T cell subpopulations involved in cytotoxic precursor or cooperator activities, since the activity of the recovered population equalled that of whole spleen when expressed as a function of the number of T cells in the experiments.

Although the basis for the selective retention of Ig-bearing cells on these nylon wool columns is not known, the procedure we have described here provides a rapid and effective method for the isolation of functional T cells from mouse spleen and lymph nodes for studies of peripheral T cell function.

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