

SEPARATION OF HELPER AND SUPPRESSOR T
LYMPHOCYTES
ON A FICOLL VELOCITY SEDIMENTATION GRADIENT*

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The existence of subpopulations of T cells has been recognized in a number of experimental models. Raff and Cantor (1) first subdivided T cells into T₁ and T₂ according to an antigen-driven maturation scheme. T₁ cells which were immature, short-lived, and noncirculating upon encounter with antigens developed into mature T₂ cells which were long-lived, circulating, and had a lower density of Thy-1 antigen on the cell surface. More recently it would appear that the T₁ and T₂ progression may occur in a number of parallel T-cell subpopulations and it may be more appropriate to subdivide T cells on a functional basis into T_H, the helper cells carrying Ly-1 antigens and T_{C,S}, the cytotoxic and suppressor T cells carrying the Ly-2,3 antigens (references 2-4 and H. Cantor, personal communication). Although cytotoxic and suppressor T cells have been separately detected in many different systems (5-8), their relationship to each other remains unclear and they are tentatively grouped together. It should also be noted that cell cooperation is not limited to T- and B-cell systems. Collaboration between T-cell subpopulations in the generation of effector T cells has been suggested in a number of experimental systems (e.g. Asofsky et al. (9); Bach and Bach (10)).

While the helper and cytotoxic functions of T cells are now more or less established concepts (5, 11), the functional role of T-suppressor cells is less well defined. Gershon (6) was among the first to observe that cells from mice made tolerant to sheep erythrocytes (SRBC) when transferred to normal mice conferred tolerance on the recipient. This "infectious" phenomenon could be completely abrogated by anti- θ treatment. In other systems, Herzenberg and Herzenberg (12), Baker et al. (13), Tada and Takemori (14), Rich and Pierce (7), and Dutton (15) provide further evidence for the existence of suppressor cells. On the other hand, Coutinho and Möller (16) have proposed a "one nonspecific signal hypothesis" and suggested that suppression is merely due to too much help. The answer as to whether helper and suppressor T cells are distinct subpopulations will help resolve this dilemma.

We have previously shown that the incubation of mouse spleen cells with concanavalin A (Con A)¹ induced both helper and suppressor activities for the

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¹ Abbreviations used in this paper: Con A, concanavalin A; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; [³H]TdR, tritiated thymidine; 2-ME, 2-mercaptoethanol; rabbit anti-BA antiserum, rabbit antimouse brain-associated θ -antigen antiserum.

humoral immune response (15, 17) and have defined the properties of the cells mediating these functions. The suppressor activity appears to be radiosensitive before induction and can be removed by treatment with goat antimouse brain-associated antigen antiserum and complement (C) while helper activity is relatively resistant to both these treatments. Both activities are absent from the spleen of nu/nu mice. These differences and the fact that the two activities can be shown to compete with one another (18) suggested that the two effects are mediated by two separate T-cell populations. In the present report we further show that cells mediating the suppressive and helper activities can be physically separated on a velocity sedimentation gradient. Helper activity is associated with a population of small, slowly sedimenting cells which have incorporated little thymidine during the induction period. Suppressor activity is associated with a population of more rapidly sedimenting cells that contain blast cells and the bulk of the incorporated thymidine. Both activities are Con A-induced and both are antimouse-brain-associated θ -antigen and C sensitive.

Materials and Methods

Mice. (C57BL/6 female \times DBA/2 male) F_1 hybrid mice were bred in our own colony. 10- to 14-wk-old male and female BDF₁ mice were used.

Chemicals and Reagents. Con A twice crystallized (lot 111) was obtained from Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill. Ficoll was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The Hypaque (50% wt/vol) was from Winthrop Laboratories, Division of Sterling Drug Inc., New York. SRBC were obtained from the Colorado Serum Co., Denver, Colo. RPMI-1640 from Grand Island Biological Co., Grand Island, N. Y., supplemented with 5% fetal calf serum (FCS) was used as culture medium. The same lot of FCS was used throughout these experiments and was obtained from International Scientific Industries Inc., Cary, Ill. [³H]thymidine (sp act 20 Ci/mmol) was from New England Nuclear, Boston, Mass.

Cultures. The pregradient cultures were carried out in Falcon no. 2005 polypropylene tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Mouse spleen suspensions were cultured at 5×10^6 cells/ml in Eagle's minimal essential medium (MEM) or RPMI by a modification of the method of Mishell and Dutton (19). Con A at 2 μ g/ml was added at time 0. Cells were cultured for 30-40 h before being subjected to separation procedures. The second culture period was done on Falcon no. 3040 microtissue culture plates. 0.05 ml of fractionated cells plus 0.05 ml of normal spleen cells were used per well. Eight replicates were done. One drop of 0.04% SRBC suspension from a 22 gauge needle was added to each well. The response was assayed by the direct hemolytic plaque assay 4 days later.

Nylon Wool Column-Passed Cells. Procedures for the enrichment of T cells were essentially those of Julius et al. (20). Briefly, prepacked nylon columns were first washed with complete media (RPMI-1640 with HEPES, 5% FCS, and 5×10^{-5} 2-mercaptoethanol [2-ME]) and incubated in gassed and sealed holders at 37°C for greater than 1 hour. The columns were then flushed with a few milliliters of warm complete media and 2 ml/column of cell suspensions at a concentration of $1-1.5 \times 10^8$ cells/ml were loaded onto the columns dropwise. The columns were again sealed and incubated for another 45 min at 37°C. After incubation, cells were eluted with warm complete media, spun down, and counted.

Removal of Dead Cells. A modified Ficoll-Hypaque flotation gradient (reference 21 and footnote 2) was used. Briefly, a dispersion medium consisting of 6 mM sodium acetate and 0.25% gelatin in Hanks' balanced salt solution (HBSS) was used to resuspend cells to $4-5 \times 10^7$ cells/ml. 1

² Swain, S., F. Modabber, and A. H. Coons. 1976. Characterization of T and B antigen-binding cells for β -galactoside. I. β -Galactosidase binding cells in the thymus and spleen of normal mice. *J. Immunol.* In press.

ml of this cell suspension was layered over 3 ml of Ficoll-Hypaque solution in Falcon no. 2001 tubes. The Ficoll-Hypaque solution was 4.5% Ficoll and 15% Hypaque in double-distilled water. The interface was gently stirred with a Pasteur pipette to prevent cells from crushing against the interface when they floated (Shortman, Cell Separation Workshop, UCLA, 1974). The tubes were centrifuged at 100 *g* for 20 min. Viable lymphocytes formed a diffused white band at the interface while most of the dead cells went to the bottom of the tube. The viable cell band and the medium above it was sucked out with a Pasteur pipette. The pool collected from several such tubes was diluted with half the volume of HBSS. Cell viability was determined by trypan blue dye exclusion. Usually the viability of a 40 h culture in the presence and absence of Con A was about 45–60% and 30–40%, respectively. The Ficoll-Hypaque procedure not only brings the viability up to 88–95% but also removes most of the erythrocytes.

Ficoll Gradient. 20% wt/wt Ficoll in HBSS was first prepared and 5% Ficoll was made from it by dilution with HBSS. Ficoll has been shown to be nontoxic for human cells (22). The technique applied is similar to those described by Everson et al. (23). A 30 ml 5–20% Ficoll preformed gradient was generated using a two-chamber gradient maker with the 20% chamber constantly stirred. Beckman 1 × 3½ inch cellulose nitrate tubes (Beckman Instruments, Fullerton, Calif.) were sterilized and coated with FCS before use. 3 ml of 5% Ficoll was then layered on top of the gradient to prevent the “streaming effect” during centrifugation. Finally, 1 ml of a 3–5 × 10⁷ cells/ml cell suspension was layered on top of the 5% Ficoll zone. Centrifugation was carried out in an International centrifuge model PR-6 (International Equipment Co., Needham Heights, Mass.) at 700 rpm for 30 min at 4°C. 1-ml vol of solution were serially removed from the top with a marked Pasteur pipette. In ³H-labeled experiments, 0.1 ml from each fraction was saved for assay. In certain later experiments, the rest of the fractions were pooled into I, II, III, and IV. The pools were then diluted with HBSS as follows: I with ½ volume, II with 1 volume, III with 1½ volumes, and IV with 2 volumes. A final centrifugation at 2,000 rpm for 20 min brought the cells down. The cells were resuspended to the appropriate concentrations with culture medium. The total cell recovery varied from 55–75%.

Rabbit Antimouse Brain-Associated θ -Antigen Antiserum (Rabbit Anti-BA Antiserum) plus C Treatment. T cell depletion from fractionated pools was achieved with anti-BA antiserum plus guinea pig C. The antiserum was prepared and absorbed according to the methods of Golub (24). Fractionated cells were resuspended to 5 × 10⁷ cells/ml with an appropriate dilution of the antiserum in HBSS. The cells were incubated for 30 min at 37°C, washed once, and resuspended to 0.5 ml of an appropriately diluted C solution. The cells were incubated for another 30 min at 37°C, washed three times, and resuspended in complete medium.

³H Labeling. 5 × 10⁶ cells/ml were cultured in tubes. After 20 h, 1 μ Ci of tritiated thymidine (³H]TdR) (diluted to a sp act of 0.24 Ci/mmol) in 0.1 ml of HBSS was added to each tube and they were incubated for another 20 h. After cell separation procedures, radioactivity was assessed using an automatic sampling harvester (25). The filters were collected, dried, and counted in a liquid scintillation counter.

Results

Cell Separation on the Gradient. Fig. 1 is a composite figure summarizing the results from several experiments which are highly reproducible. It shows the linearity of the gradient, the distribution of lymphoid cells, and the distribution of cells that incorporated [³H]TdR (present during the last 20 h of culture) in the presence and absence of Con A. The bulk of the thymidine-containing cells sedimented more rapidly than the total cell peak. This radioactive peak was not due to aggregates of labeled small cells since there was no change in the profile when 10 mg/ml α -methyl-D-mannopyranoside was included in the gradient (data not shown). Moreover, when cell suspensions were checked under the microscope, no aggregates were found.

Examination of stained smears showed that fractions 1–13 (pool I, see below) contained predominately small cells while most of the big cells were confined to fractions 21–34 (pools III and IV). The middle fractions 14–20 (pool II) had cells of

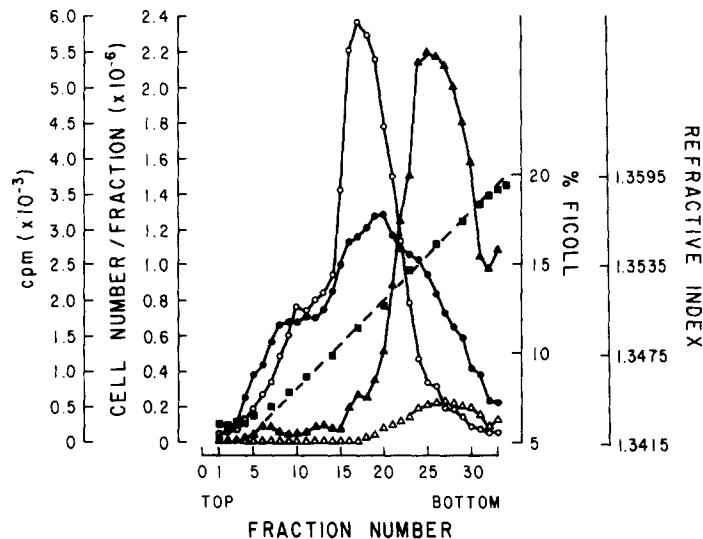


FIG. 1. Cell number profile, ^3H profile, and linearity of Ficoll gradient. Cells from a 40 h culture with or without Con A (final concentration $2 \mu\text{g}/\text{ml}$) were separated on a 5–20% Ficoll gradient and fractionated (Materials and Methods). The fractions were assayed for cell number and [^3H]TdR uptake. The refractive indices of fractions without cells were also determined. Previously, a standard curve relating refractive index to percent Ficoll was constructed. (○—○), cell number in the absence of Con A; (●—●), cell number in the presence of Con A; (△—△), ^3H counts in the absence of Con A; (▲—▲), ^3H counts in the presence of Con A; (---), theoretical linear gradient; and (■), observed refractive indices.

all sizes. Similar results were obtained when the pooled fractions were analyzed by a Coulter Counter Particle Size Distribution Plotter (Coulter Electronics Inc., Hialeah, Fla.). The tritium peak and the bulge on the leading edge of the whole-cell curve were absent when the spleen cells had been cultured in the absence of Con A.

Functional Differences of Fractionated Cells. Fractions from the gradient were pooled to give four pools: pool I (fractions 1–13), pool II (fractions 14–20), pool III (fractions 21–25), and pool IV (fractions 26–34). (In later experiments indicated below, pool III was made from fractions 18–23 and pool IV from 24–34.) The cell suspensions in these pools were assayed for their effects on the primary response of fresh normal spleen cells to SRBC. The protocol for these experiments is indicated in Fig. 2. The medium for the postgradient cultures but not the pregradient cultures contained 5×10^{-5} M 2-ME. Modifications of this standard protocol for specific experiments will be indicated in the respective experiments. The gradient separated cells were irradiated before their addition to the normal cells. We have shown that both helper and suppressor activity of effector cells are relatively radioresistant after induction (17). Irradiation is thus a convenient way to eliminate any contribution of B-cell activity to the response by the cells whose T-cell activity is being assayed. The results of a typical experiment are shown in Fig. 3. When graded numbers of irradiated pool I cells were added, the response of normal cells was increased while irradiated cells from pool IV were clearly inhibitory. The effects of pool II and III and the

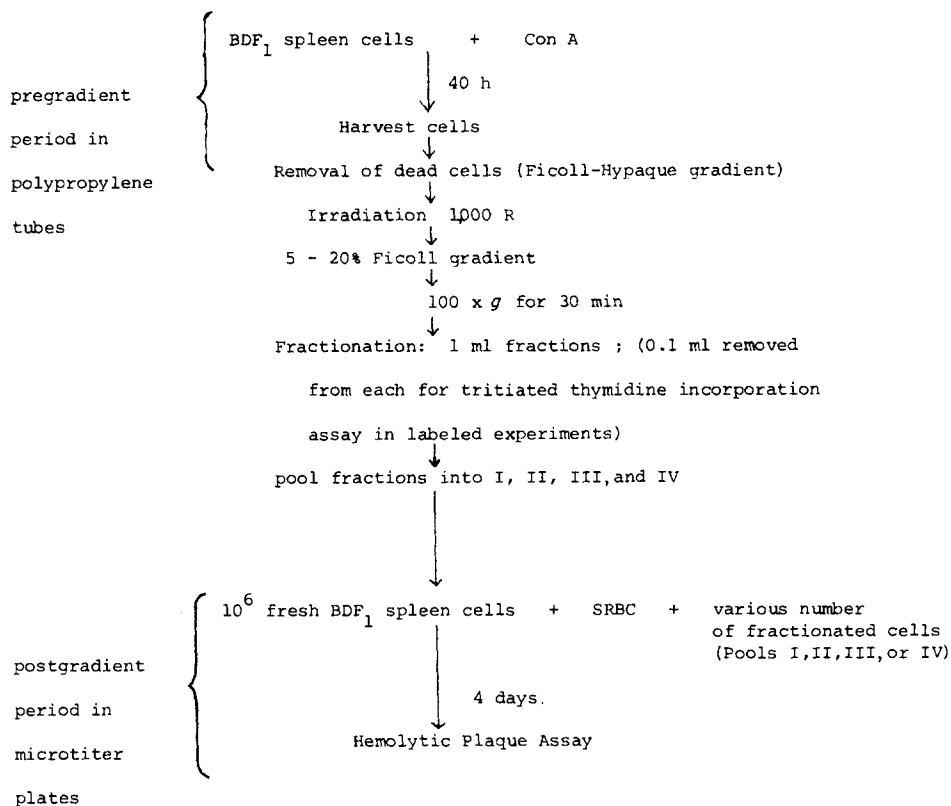


FIG. 2. Basic experimental scheme.

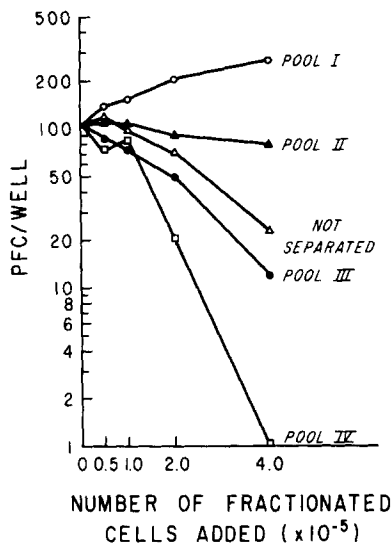


FIG. 3. Functional differences of fractionated cells. Various number of fractionated cells were titrated into 10^6 normal spleen cells plus SRBC. Plaque assay to SRBC was determined 4 days later. Each point represents the arithmetic mean of eight replicates. The cells were from a 40 h culture. Pool I, fractions 1-13; pool II, 14-20; pool III, 21-25; and pool IV, 26-34. PFC, plaque-forming cells.

unseparated population are also illustrated. This pattern of activities was reproducibly observed in many experiments.

T-Cell Dependency. It is important to determine whether T cells are responsible for the observed activities. Two different approaches were adopted. Firstly, T cells were depleted from the fractionated pools by anti-BA antisera plus C treatment. As shown in Fig. 4, both activities are sensitive to such treatments. Next, we used nylon wool column-passed cells, which were enriched for T cells, for the pregradient cultures. Since the column also removed most of the macrophages, 2-ME was added to the culture media for the pregradient culture as well as in the postgradient culture. Fig. 5 shows that the activities were retained in the cell preparation that passed through the column. It would thus appear that T cells are required for the mediation of both the helper and suppressor activities.

Con A Dependency and Con A Carry-Over. Recently Burns et al. (26) reported that suppressor T cells could be generated by culturing normal spleen cells for 4 days. In our system, the pregradient culture period is 30-40 h and the generation of both T helpers and T suppressors was found to be Con A dependent. As can be seen in Fig. 6, when Con A was omitted in the pregradient culture period, no activities were observed.

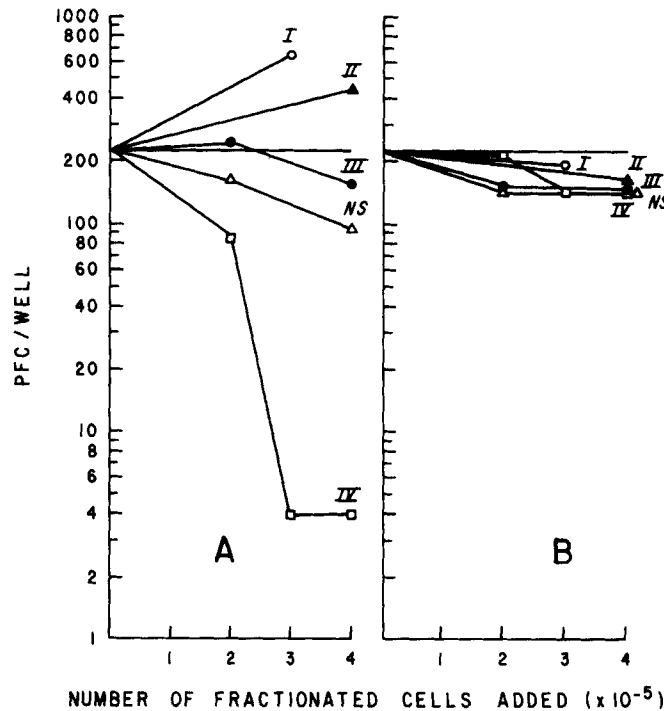


FIG. 4. Effects of rabbit anti-BA antiserum treatment of fractionated cells. Experimental procedures were the same as in Fig. 3 except that half of each cell pool was treated with rabbit anti-BA antiserum (as in B) and the other half untreated (as in A) before titration into normal spleen cells. Pool I, fractions 1-12; pool II, 13-17; pool III, 18-23 and pool IV, 24-34. PFC, plaque-forming cells.

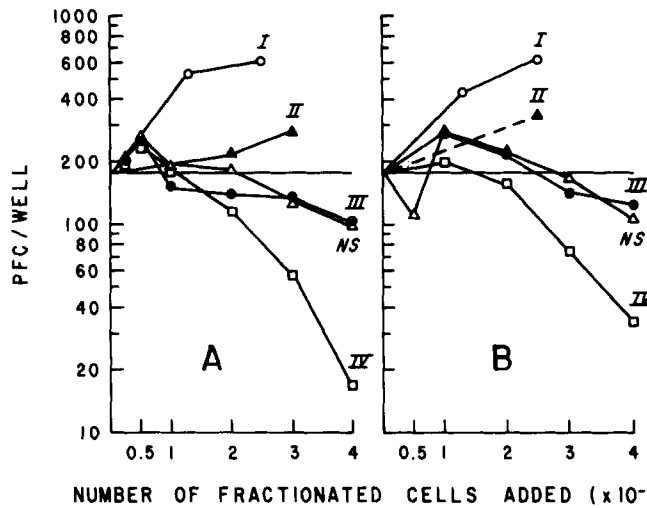


FIG. 5. Titration of fractionated cells from nylon wool column-passed cell cultures. Experimental procedures were those of Fig. 2. (B) nylon wool column-passed cells were used in the pregradient cultures. The responses of fractionated normal spleen cells were included for comparison (unpassed) (A). Pool fractions were the same as in Fig. 4. PFC, plaque-forming cells.

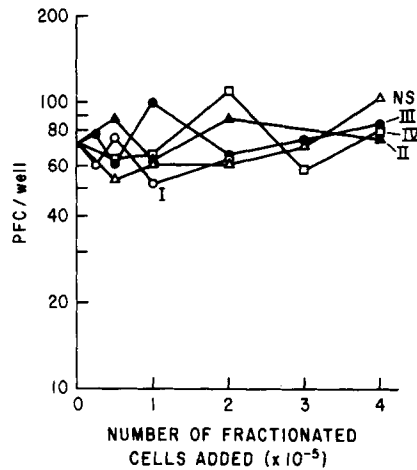


FIG. 6. Titration of fractionated cells with the omission of Con A in the pregradient culture period. Experimental procedures were those of Fig. 2 except that no Con A was added to the pregradient cultures. Pool fractions were the same as in Fig. 4. PFC, plaque-forming cells.

It could be argued that the helper and suppressor activities were due to carry-over of Con A from the pregradient cultures. The data presented in Table I rules out this possibility. In this experiment, fractionated cells were added to normal cells with or without α -methyl-D-mannopyranoside at a final concentration of 2 mg/ml. The presence of the sugar does not abrogate either the helper or suppressor effect. In addition, we found that Con A concentrations in the range of 0.5 μ g/ml to 1 ng/ml did not induce any helper or suppressor functions (data not presented).

TABLE I

Pool	No. of fractionated cells added ($\times 10^5$)	PFC/well	
		- Mannose	+ Mannose
Control (no addition)	0.0	157	167
I	2.5	527	481
	1.5	375	397
II	4.0	287	271
	2.0	204	196
	1.0	156	140
III	4.0	159	175
	3.0	260	189
	1.5	276	154
IV	4.0	44	37
	2.0	130	129
	1.0	213	175
Unseparated cells	4.0	115	98
	2.0	231	145
	1.0	211	151

Titration of helper and suppressor activities in the presence and absence of α -methyl-D-mannopyranoside. Cell pools were the same as in Fig. 4. Various numbers of fractionated cells were titrated into 10^6 normal spleen cells plus SRBC with or without α -methyl-D-mannopyranoside at a final concentration of 2 mg/ml. Plaque assay to SRBC was determined 4 days later. Each number represents the arithmetic mean of eight replicate wells. The cells were from a 34 h culture. PFC, plaque-forming cells.

Demonstration of the Stimulatory Effect. Experiments presented so far demonstrate marked inhibitory activity associated with pool IV but only a two- to threefold stimulatory activity in pool I. In the experimental protocol, almost an optimal number of cells are used in the indicator population. An inhibitory effect can be readily observed but stimulation is minimal since the B cells are already responding well in the presence of adequate T help. In addition, it was felt that T_H cells may have been induced earlier than $T_{C,S}$ cells and after 40 h in culture, the wave of stimulatory activity might have passed while the inhibitory activity is still high. Further experiments were therefore performed with the following modifications: (a) the pregradient culture period was reduced from 40 to 30 h and (b) fractionated cells were titrated into 4×10^5 rather than 1×10^6 normal cells per well. Fig. 7 shows that the stimulatory effect of the upper fractions can be more readily assayed when fractionated cells are added to a small number of normal cells (4×10^5 as in Fig. 7 A) which are deficient in helper T cells.

Discussion

In view of the complexity of T-cell functions in the regulation of the immune response, we attempted to apply physical methods to isolate subpopulations of

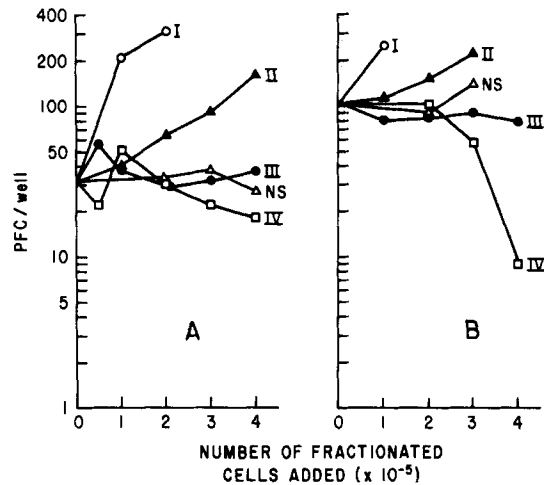


FIG. 7. Titration of fractionated cells at two different cell densities. Experimental procedures were those of Fig. 2 except that in A, 4×10^5 normal cells per well and in B, 1×10^6 normal cells per well were used. The fractionated cells were from a 30 h culture. Pool fractions were the same as in Fig. 4. PFC, plaque-forming cells.

cells and to study their mode of action separately. The results in this report indicate that velocity sedimentation centrifugation (27) is a useful tool (28) for separating and analyzing the T-cell subpopulations examined here. The experiments presented show that Con A-induced helper and suppressor activities in the humoral response to SRBC are mediated by two distinct subpopulations of T cells separable on a 5–20% Ficoll gradient. In a 40 h spleen culture with Con A, helper activity is mainly a function of a population containing small cells while a population containing blast cells exhibits suppressive activity (Fig. 3). The absolute levels of stimulatory or inhibitory activity can vary, but the general pattern of Fig. 3 is always preserved.

We, as well as many others (6, 13, 15) have consistently observed that both the helper and suppressor activities are T-cell dependent (Fig. 4). The fact that helper and suppressor T cells can be separated from the same initial *in vitro* culture strongly supports our earlier contention that helper and suppressor cells are distinct T-cell subpopulations. The same conclusion has been reached independently by Gerber and Steinberg (29) who separated helper and suppressor thymocytes utilizing unit gravity sedimentation technique. However, in their study, the suppressor cells were the slowly sedimenting cells while the helper cells sedimented rapidly. The cells were obtained from a different source, thymus from 1-mo-old NZB/W mice and the ability of the fractionated cells to help or suppress the generation of graft-vs.-host disease by $4\frac{1}{2}$ -mo-old and 12-mo-old NZB/W mice in Swiss neonates was assayed. Since the maturation patterns of T cells after antigen or Con A stimulation are poorly understood and may be different, the above observation and ours may not be incompatible. In fact, our preliminary study with an antigen-specific system indicates that antigen-stimulated helper cells are in the faster sedimenting populations.³

The recently adopted nomenclature of T_H for helper cells and $T_{C,S}$ for cytotoxic

³ Swain, S., and H. Tse. Manuscript in preparation.

and suppressor cells is largely based on results of studies of Ly surface antigens. T_H cells are Ly-1 positive, possibly recognize *I*-region "LD" antigens in primary cell-mediated immunity reaction and provide nonspecific T-T help in mixed lymphocyte reactions. $T_{C,S}$ are Ly-2,3 positive and are among the precursor and effector cytotoxic cells recognizing H-2K and H-2D "SD" antigens (2). The characterization of our separated helper and suppressor populations in terms of Ly antigens is in progress.

It is unlikely that the suppressor activity is due to cytotoxic cells (30) rather than suppressor cells because recultured pool I and pool IV cells could produce stimulatory and inhibitory supernates, respectively (unpublished observations). Suppressive supernates from Con A-activated *in vitro* spleen cell cultures have also been obtained by Rich and Pierce (31).

Preliminary experiments have shown that similar T_H and $T_{C,S}$ cells can be generated and separated from 48-h cultures of allogeneic mixtures of mouse spleen cell suspensions. This lends additional support to the notion that the observed activities are not simply due to carry-over of Con A from the pregradient culture period to the postgradient period.

A final interesting point that emerges from the present study is the relationship between the incorporation of [3H]TdR and the generation of T_H and $T_{C,S}$ cells. Since T_H activity is always associated with small cells which remain at the top of the gradient, it would appear that the generation of T_H does not require blast transformation or cell division. On the other hand, $T_{C,S}$ cosediment with actively DNA-synthesizing blast cells towards the bottom of the gradient. The possibility that the small cells which exhibit helper activity are the progeny of cells which have divided seems unlikely because very little incorporated thymidine is found in the small cell population (Fig. 1) and T_H cells are radioresistant even before induction.

Finally, it is important to realize that the T-cell functions that we have examined here are the Con A-induced helper and suppressor activities. The relationship of these activities to the analogous activities induced by antigen or alloantigens has not been established.

Summary

A 5-20% Ficoll velocity sedimentation gradient has been successfully applied to separate concanavalin A (Con A)-induced helper; and suppressor T cells. When titrated into a constant number of fresh normal spleen cells responding to sheep erythrocytes, cells from the top pool show stimulatory effects while those from the bottom pool show inhibitory activity. Both activities are found to be Con A dependent and anti- θ sensitive. We conclude that Con A-induced helper and suppressor T cells are distinct subpopulations and such separation will allow further characterization of these cell types.

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