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MECHANISM OF ALLOTYPE SUPPRESSION*

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Presented at Symposium on Suppressor Cells in Immunity

The University of Western Ontario
London, Ontario, 72, Canada

May 12-14,1975

*Research supported in part by NIH Grants HD-01287, CA 04681, and GM-17367.

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Once upon a time, (and not so long ago), the idea that T cells played an important role in the regulation of immune responses was considered about as realistic as Galileo's earth moving around the sun, and studying these elusive beasts was considered fit fare for women and incompetents. Not so any more! As the program of this conference amply demonstrates, the number of systems in which suppressor cells have been shown, and the number of laboratories now working with suppressive effects has reached the point where "suppressology" threatens to become a discipline unto itself. If so, perhaps we should offer Richard Gershon the first chair, since with his amazing peripatecity he has certainly worked on more different suppressive effects than all of us combined.

Seriously, it is good to meet here to discuss the many instances of regulation of immune responses by suppressor cells. The chairmen are to be commended for bringing us all together. Perhaps, if we are lucky, the joining of the different points of view afforded by the variety of systems under study will lead to some general insights into how suppressor cells are induced, how they regulate responses, and how they themselves are regulated.

We have been studying a long-lived suppressor T cell population which regulates over-all immunoglobulin production as well as specific antibody production of one of the immunoglobulin allotypes in an allotype heterozygote (1). This population is induced (or expanded) in SJL x BALB/c mice by perinatal exposure of this particular hybrid to anti-allotype antibody. Once induced, it is maintained for the life of the animal, without further antibody exposure. In the majority of exposed animals at least some circulating allotype is produced while the animals are between 12 and 20 weeks of age but after that time the suppressor cells exert their full effect and circulating allotype is generally undetectable.

Suppressor cells with similar properties have recently been found in bone marrow of agammaglobulinemic chickens (2) and in human peripheral blood T

lymphocytes taken from patients with common variable agammaglobulinemia (3). The negative regulation of immunoglobulin production in these two cases bears a striking resemblance, as far as it has been followed, to the chronic allotype suppression we observe in SJL x BALB/c mice. These parallels suggest that cells analogous to allotype suppressors may be more ubiquitous than we originally imagined, but whether allotype suppression is a useful model for the human disease is still to be determined, as is the significance of allotype suppressor T cells in the normal regulation of immunoglobulin production.

One of the key questions regarding the mechanism of allotype suppression is at what stage suppressor T cells interrupt the B cell pathway which normally culminates in Ig-1b producing cells. We have shown that when SJL x BALB/c mice are completely suppressed for Ig-1b allotype production, they have no detectable Ig-1b in serum, no Ig-1b producing plasma cells in spleen or lymph nodes, and only a very low probability of regaining the ability to produce circulating Ig-1b except in small amounts for relatively short periods of time (1,4). Nevertheless we have found recently that when primed with antigens, these mice develop normal Ig-1b memory which is demonstrable in an adoptive secondary response if the suppressor T cells are removed prior to transfer (5). Furthermore, they have normal numbers of Ig-1b bearing lymphocytes (4,5) which, when isolated from antigen primed suppressed mice, contain essentially all of the memory B cells which give rise to Ig-1b plaque forming cells (PFC) but no memory B cells for other IgG-PFC (5). These data (which are presented in shortened form in the sections which follow), narrow the locus of suppressor cell attack to the latter part of the B cell differentiation pathway, somewhere after the appearance of the Ig-1b bearing memory cells but before differentiation of these cells proceeds to antibody producing cells.

Development of Ig-1b Memory to DNP in Suppressed Mice

In the adoptive secondary response to dinitrophenol (DNP), mature precursor (memory) B cells differentiate to cells producing large amounts of IgG antibody reactive with DNP(DNP-PFC). The differentiation of the DNP memory cells, obtained here from spleens of mice primed with DNP-Keyhole Limpet Hemocyanin (DNP-KLH), depends both on restimulation with the antigen and the cooperation of T cells which recognize the (KLH) carrier. Virtually all of the IgG DNP-PFC found in spleens of heavily irradiated recipients seven days after transfer are progeny of the transferred memory cells (6).

The data in Table 1 show that normal numbers of memory cells capable of giving rise to Ig-1b DNP-PFC are present in spleens of completely suppressed mice primed with DNP-KLH. These Ig-1b memory cells are demonstrable in the DNP adoptive secondary response when suppressor T cells are depleted prior to transfer. As the data show, without T depletion, transferred spleen cells from primed suppressed mice give rise to Ig-1a DNP-PFC (the allelically determined γG_{2a} antibody) and other IgG DNP-PFC but no detectable Ig-1b DNP-PFC. T depleted (by anti Thy-1 + complement) spleen cells transferred alone give no IgG DNP-PFC response at all because the anti Thy-1 removes cooperator T as well as suppressor T cells. But KLH (carrier) primed T cells restore the response of these T depleted cells. The number of Ig-1b DNP-PFC obtained differs only slightly from that obtained from primed normal (non-suppressed) donors.

Table 1

DEVELOPMENT OF Ig-1b MEMORY FOR DNP IN CHRONICALLY SUPPRESSED MICE

Group No.	DNP-KLH Primed Spleen Cells Transferred ($\times 10^6$) Suppressed		Normal	Indirect DNP-PFC*		
	Untreated	T Depleted	Untreated	Ig-1b	Ig-1a	Total IgG
1	10	--	--	0	670	5,220
2	--	5 + T_{coop}**	--	560	510	4,230
3	--	5	10	1,950	1,780	7,880
4	--	--	10	1,200	1,050	5,880

*Indirect DNP-PFC/10^6 recipient spleen cells. Direct PFC < 40.

**Supplemented with 10×10^6 KLH primed spleen cells.

The rough equality of the Ig-1a and Ig-1b responses observed in the carrier T supplemented recipients of splenic B cells from the DNP primed suppressed mice (see Table 1) is the best indication that suppression does not interfere with memory development. Since these responses are always just about equal in DNP-KLH adoptive secondary recipients of spleen from primed normal mice, comparing the Ig-1b response to the Ig-1a within the same group of animals provides an internal reference standard which is independent of differences in the magnitude of priming or in the effects of cell manipulations which can occur between groups. Thus priming with DNP-KLH leads to development of normal Ig-1b memory regardless of the presence of large numbers of suppressor T cells.

If suppressor T cells do not interfere with Ig-1b memory development, then they must prevent allotype synthesis by acting on a later stage in the B cell differentiation pathway. This makes the suppression observed in the intact animal similar to suppression observed in transferred mixtures of unprimed suppressed cells and primed normal cells. In both cases, B cell differentiation pathway is interrupted between the memory cell and the producing cell. Therefore the characterization of the cells which carry Ig-1b memory takes on considerable importance with respect to determining the mechanism of allotype suppression.

Memory B cells, according to current theory (7), are IgG bearing lymphocytes committed to differentiate to cells producing large amounts of the IgG molecule displayed on the lymphocyte surface. Thus Ig-1b memory cells should have surface Ig-1b, and suppressed animals, since they have normal numbers of Ig-1b memory cells, should have normal numbers of Ig-1b bearing cells. The data in the following experiments show this to be the case.

Presence of Ig-1b Bearing Cells in Suppressed Animals

To determine the percentage of Ig-1b bearing cells, spleen cell suspensions from individual animals were incubated first with an absorbed rabbit antiserum specific for Ig-1b (kindly provided by Dr. John Coe) and then with fluorescein conjugated goat anti rabbit immunoglobulin antibodies. Under the conditions

used, only those cells which bind the first step reagent (anti Ig-1b) become visibly fluorescent with the fluorescence microscope. (The proof that the reagent is specific for Ig-1b is best demonstrated by separation data in the next section). To facilitate microscope counting, since less than 1% of the cells are Ig-1b positive, the fluorescent cells were enriched 10-fold by using the Fluorescence Activated Cell Sorter (FACS) prior to making smears.

The data in Table 2 show that no siginificant differences exist between the number of Ig-1b bearing cells found in spleens of suppressed mice and spleens of normal syngeneic control mice never exposed to antiallotype antibody. In both cases, between 0.5 and 0.6% of the spleen cells have surface Ig-1b.

Table 2

PERCENTAGE OF CELLS WITH SURFACE Ig-1b IN NORMAL AND SUPPRESSED MICE

SJL x BALB/c 8-10 months	Number of Animals	Serum Ig-1b mg/ml	Ig-1b Bearing Cells* %
Normal Spleens	8	> 0.5	0.54 ± 0.15
Suppressed Spleens	7	< 0.01	0.59 ± 0.11

*Spleen cell suspensions stained with rabbit anti Ig-1b and then with F-goat anti rabbit. Cells were enriched ten-fold by passage through FACS prior to counting with fluorescence microscope.

Presence of Ig-1b Memory Cells in Populations of Ig-1b Bearing Cells Isolated from Spleens of Suppressed Mice

Ig-1b bearing cells from anti Thy-1 plus complement treated DNP-KLH primed suppressed spleen were made fluorescent by the staining procedure described in the preceding section. The suspension containing the fluorescent cells was then passed through the FACS twice. In the first pass, the brightest 10% and the dullest 85% were saved. In the second pass, the brightest 10% of the cells were further separated to obtain a cell fraction which contained the brightest 10% of cells in the fraction, thus the brightest 1% of the original anti Thy-1 killed spleen suspension. (All of the visibly fluorescent (Ig-1b bearing) cells were in this fraction.) The two fractions of separated cells, i.e., the brightest 1% (enriched for Ig-1b bearing cells) and the dullest 85% (depleted for Ig-1b bearing cells), were supplemented with KLH primed spleen cells from normal (non-suppressed) donors to provide an excess of cooperator activity, and tested for precursor activity in the adoptive secondary response to DNP-KLH. Dosages of transferred cells were adjusted so that the number of isolated bright or dull cells transferred would be equal to the number of those cells transferred in the original unseparated suspension.

The data in Table 3 show that the isolated fraction enriched for Ig-1b bearing cells, comprising only 1% of the unseparated cells, contained roughly two-thirds of the Ig-1b memory cells formed in the unseparated spleen suspen-

Table 3

PRESENCE OF Ig-1b MEMORY IN Ig-1b BEARING CELLS FROM CHRONICALLY SUPPRESSED MICE

DNP-KLH Primed Spleen Cells Transferred ($\times 10^6$)									
	Suppressed		Non-Suppressed	Indirect DNP-PFC*				
Description	Total Cells	Ig-1b Bearing Cells Contained	Total Cells	Total IgG	Ig-1b	Ig-1a	Ig-1a + Ig-4a	Ig-G_1
Untreated	10		--	4,560	0	471		
--	--		10	3,450	440	456		
Untreated	10		10	6,980	0	785		
T depleted	5		10	7,020	810	791		
T depleted stained for Ig-1b **	5	~ 0.04	--	2,970	335	356	1,510	980
Dullest 85%**	5	< 0.0005	--	2,620	31	310	1,320	870
Brightest 1%**	0.05	0.045	--	185	198	0	0	0

*Indirect DNP-PFC/10^6 recipient spleen cells. Direct PFC < 40.

**Supplemented with 10×10^6 KLH primed spleen cells. "T depleted" cells were prepared by treating spleen cells from DNP-KLH primed mice with Thy-1 plus complement. Fluorescent Ig-1b cells were obtained by staining the T depleted cell suspension first with rabbit anti Ig-1b, then with F goat anti rabbit IgG. After staining, the cells were passed through the FACS to obtain separated fractions.

sion. This fraction, however, did not contain memory cells for DNP-PFC producing antibody of any other immunoglobulin class. In sharp contrast, the fraction depleted for Ig-1b bearing cells (dullest 85%) contained less than 10% of the Ig-1b memory cells in the unseparated suspension. Instead, it contained essentially all of the other IgG memory cells.

Thus, in spleens of DNP-KLH primed suppressed mice, the memory for Ig-1b is carried by Ig-1b bearing cells and these cells are committed exclusively to the production of Ig-1b, at least within the confines of the adoptive secondary response.

Mechanism of Allotype Suppression

The co-existence of suppressor T cells and Ig-1b memory B cells in spleens of suppressed mice suggests strongly that the mechanism of suppression does not involve the killing of resting memory cells, although it does not preclude B cell killing if the memory cells are turning over rapidly or if killing occurs at a later state of development towards Ig-1b producing cells. The absence of any cells with even small amounts of cytoplasmic Ig-1b (4), however, says that if suppression involves killing of B cells on their way to becoming antibody producing cells it must be at a stage very shortly after the memory cell is triggered. As yet we have not clearly excluded these possibilities; however, evidence is now accumulating which suggests a completely different role for allotype suppressor T cells.

Some time ago, we showed that an antagonism exists between suppressor and cooperator T cells such that increasing the cooperator dose decreases the effect of a given suppressor dose and vice versa (8). We have now completed a series of experiments exploring this antagonism which suggest that suppressor T cells could prevent memory cell differentiation by specifically neutralizing cooperator T cell activity rather than by directly attacking the B cells (9). Although the data are preliminary in the sense that we have not directly shown an interaction between suprpessors and cooperators, the results of titrations of suppressors, cooperators and hapten primed B cells in the DNP adoptive secondary response appear to indicate that suppressors and cooperators stoichiometrically neutralize each other's activity independent of interaction with B cells.

Suppressor-Cooperator Antagonism

For these experiments the adoptive mixture-transfer assay was modified so that each of these three cell populations could be varied independently and titrated at limiting doses against the others. Spleen cells from short-term KLH-primed mice were used as a source of suppressor T cells. None of these cell suspensions showed any measurable IgG DNP-PFC response when transferred alone.

Dose ranges for suspensions of each cell type were established in preliminary experiments. Then in one large experiment, primed B cells, suppressors and cooperators were transferred, each at several doses, to irradiated recipients. The recipients were challenged on day 0 with DNP-KLH and, as in previous experiments, the number of Ig-1a, Ig-1b and total IgG DNP-PFC in recipient spleens were determined seven days after transfer.

The data from these experiments is too lengthy to present here in its entirety (see reference 9); however, Figures 1 and 2 show the essential argument using representative data from one experiment.

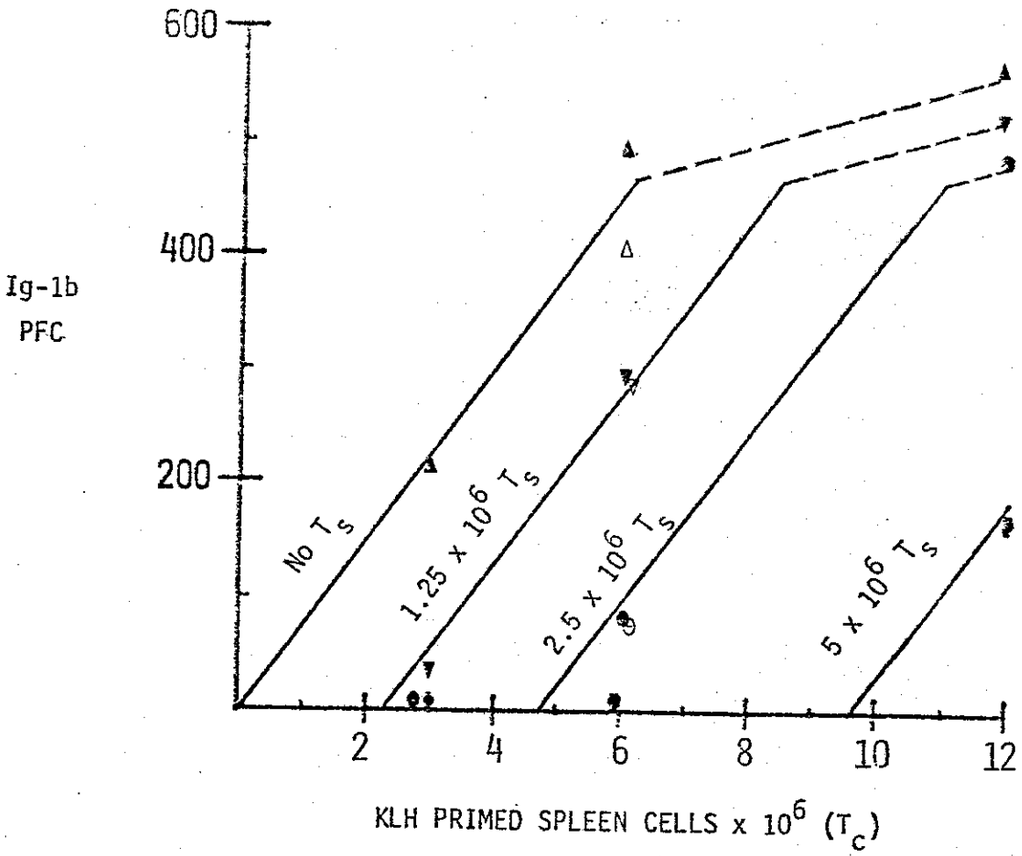

Figure 1. Effect of Suppressor T Cells on Cooperator T Cell Titration.

Ig-1b DNP-PFC (per 10^6 recipient spleen cells) normalized to 5 x 10^6 primed B cells transferred. Solid symbols are for 5 x 10^6 primed B cells (anti Thy-1 treated DNP-KLH primed spleen cells); open symbols are for 2.5 x 10^6 primed B cells normalized to 5 x 10^6 injected B cells. Data points are observed PFC responses. Curves are theoretically predicted values calculated from the equation PFC = $k \cdot (B) \cdot (T_c - \alpha \cdot T_s)$, where k and α, derived empirically, have the values 15 and 1.9 respoctively. Dotted portion of curve is above saturation for T_c.

Figure 1 shows the Ig-1b DNP-PFC response as a function of KLH primed spleen dose (T_c) in the absence and at three dose levels of suppressed (T_s) spleen cells. The data is normalized to Ig-1b PFC response/ 5 x 10^6 primed B cells transferred. This normalization puts the titration points for various T_c and T_s doses with different B cell doses on the same curve (i.e., points at a given T_c and T_s combination become roughly coincident).

The coincidence of the normalized points is shown here at only one dose of cooperator T cells and one dose of B cells. In other experiments, a similar coincidence is obtained with a wider range of each of these cell types (9), and, as in this experiment, at several doses of suppressor T cells. In all cases, the number of PFC obtained per B cell transferred is independent of B cell dose. These data suggest that the number of B cells transferred is irrelevant with respect to suppression since changing the B cell dose does not alter the cooperator titration curves in the presence (or absence) of suppressors. The extent of suppression, must depend wholly on the doses of suppressor and cooperator T cells (see Figure 1 and Ref. 9).

The solid portion of the curves drawn in Figure 1, which fit the data points quite well, are actually theoretical curves drawn from a simple equation which accurately predicts the Ig-1b PFC response in titration experiments such as the one presented in Figure 1. The form of the equation was suggested from the observation, (shown for this experiment in Figure 2), that the amount of cooperator activity lost is proportional only to the suppressor cell dose and is independent of cooperator (or primed B cell) dose. The amount of cooperator activity lost was determined by subtracting the amount of cooperator activity left (i.e., the number of carrier primed T cells which would give the observed response in the absence of suppressors) from the total number of cooperators added.

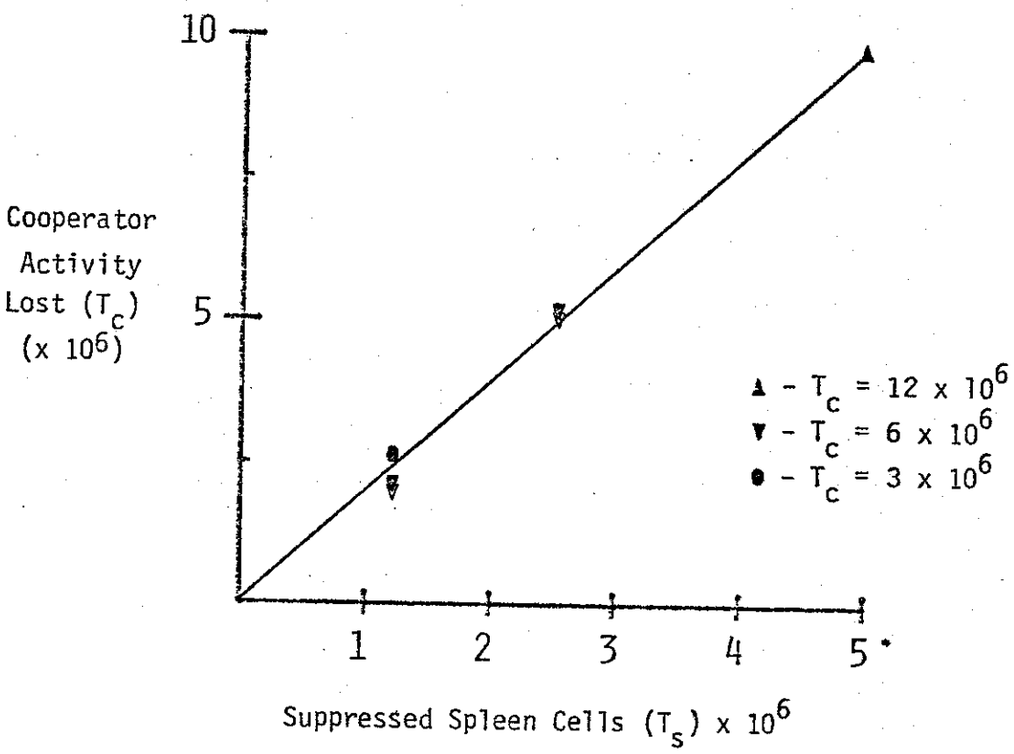

Figure 2. Loss of Cooperator Activity as a Function of Suppressor T Cell Dose. (See text for method used to calculate cooperator activity lost.)

As the data show, the cooperator loss increases linearly with suppressor (T_S) dose over the entire range of suppressor concentrations, regardless of the number of cooperator T cells (T_C) or primed B cells transferred. For example, at 1.25×10^6 T_S, the cooperator activity in approximately 2.4×10^6 T_C is lost regardless of whether the injected T_C dose was (3, 6 or 12) $\times 10^6$, or whether the B cell dose was (2.5 or 5) $\times 10^6$.

Thus we can state that, for all doses of T_C, including doses above T_C saturation,

$$(1) \qquad T_C \text{ lost} = \alpha \cdot T_S$$

where α is a proportionality constant which relates the relative effectiveness of the pools of cooperator and suppressor spleen cells used in the experiment and is equal to the slope of the line in Figure 2.

Since T_C lost was determined by subtracting the residual cooperator activity (T_C left) from the injected T_C dose, i.e.,

$$(2) \qquad T_C \text{ lost} = T_C - (T_C \text{ left}),$$

by combining equations (1) and (2) and rearranging terms, the amount of residual cooperator activity becomes predictable in terms of T_C and $\alpha \cdot T_S$. Thus,

$$(3) \qquad T_C \text{ left} = T_C - \alpha \cdot T_S.$$

The data presented in Figure 1 show that, in the absence of suppressor cells (i.e., $T_S = 0$), the PFC response per primed B cell transferred is linear with increasing T_C until T_C saturation is reached. Thus, below saturation,

$$(4) \qquad \text{PFC/(B cell)} = k \cdot T_C$$

where k, determined directly from the data, is a constant which includes the constants which correct for the actual number of memory B cells injected as opposed to the total number of T depleted primed spleen cells injected; the efficiency of conversion of memory cells to PFC; the actual number and efficiency of cooperators, etc.

If, in the presence of suppressors, the response is determined by the residual cooperator activity (T_C left), the suppressed response should be provided by the equation

$$(5) \qquad \text{PFC/(B cell)} = k \cdot (T_C - \alpha \cdot T_S)$$

as long as $(T_C - \alpha \cdot T_S)$ falls below the saturating T_C dose.

The solid portions of the curves in Figure 1 were drawn from equation (5) and show that it accurately predicts the Ig-1b PFC response at the various combinations of T_C, T_S and B cell doses used in the experiment. Out of 33 data points from this and another titration experiment described in the same publication, only three differed more than 20% from the values predicted by the equation. (For the original data for both observed and expected values in these experiments, as well as a more lengthy derivation of equation (5) and a discussion of other mathematical models, see Ref. 9). In other smaller experiments (unpublished), the predicted values also agree quite well with the data, although determination of the constants is a little less accurate.

From our experience with this equation over the last few months, we are reasonably convinced that it accurately reflects the quantitative interactions between T_C, T_S and primed B cells in the suppression of the Ig-1b adoptive secondary response to DNP. Whether it should be taken as evidence of a direct suppressor-cooperator interaction, however, is still open to question. One serious drawback to this interpretation is that suppression in mice exposed to anti Ig-1b is highly specific for Ig-1b. No suppression of either Ig-1a or other IgG classes is observed in these mice. Therefore, if the suppressor T cells suppress by removing cooperator activity, the cooperator activity being removed must be committed to cooperate only with Ig-1b memory cells!

We find it hard, at this point, to seriously propose the existence of allotype specific cooperator T cells. Data on immune deviation (10) and on cooperation in the IgE response (11) suggest that there may be class specific cooperators. But no evidence whatever supports the postulation of allotype specific helpers. In fact, to the contrary, many laboratories (including our own) have shown that cooperation clearly occurs between B cells and T cells each taken from one of a pair of allotype congenic strains (12,13). However, from existing data we cannot exclude the idea that B cell products are involved in conferring specificity on the T cell, nor can we exclude even rather large quantitative differences in the effectiveness of cooperation across an allotype barrier. Therefore, it is possible (although perhaps not too probable) that allotype specific T cells do exist.

For the present we feel that the idea that suppression is due to the removal of cooperator activity should be considered as nothing more than a provocative hypothesis which could prove to be correct. Although the equation we described is useful in predicting responses in allotype suppressor T cell experiments, its significance with respect to the mechanism of suppression must remain in question until the suppressor cooperator interaction is demonstrated directly.

Partly because we would like to be able to test the above model, but for more basic reasons as well, we have recently been putting a substantial part of our effort into experiments designed to characterize the allotype suppressor cells and, if possible, to isolate them free of other T cell types. We find that the suppressor cells pass through nylon wool columns (1), are resistant to *in vivo* cortisone treatment and are sensitive to relatively low doses (<600R) of *in vitro* irradiation (unpublished observations).

Dr. Vicki Sato in our laboratory, using the FACS, has now obtained preliminary data which indicate that the suppressor T cells are contained within a subpopulation of T cells which is larger than average and has a lower surface density of T cell antigens. (See Tables 4 and 5.)

The data in Table 4 show that the suppressor T cells are found amongst the largest 20% of nylon passed (T) cells from suppressed spleen when these cells are isolated with the FACS. (The FACS uses low-angle light scatter as a measure of size.) Virtually all of the suppressive activity in the original spleen is present in the large cell fraction, and very little, if any, in the small. Similarly, data in Table 5 show that nearly all of the suppressive activity in the nylon passed T cells is found in the dullest 30% of cells when the cells are stained with a rabbit serum which detects T cell antigens and separated by fluorescence with the FACS. (Rabbit anti moust brain anti-serum abosrbed to be specific for T cells was digested with pepsin to make

TABLE 4

ISOLATION OF SUPPRESSOR T CELLS BY FACS SCATTER (SIZE)

SJL x BALB/c Spleen Cells Transferred (x 10^6)				Indirect DNP-PFC*	
	Unprimed Suppressed Nylon T				
Normal DNP 1^0 Whole	Treatment	Total Cells	Corresponds To	Ig-1b	Total IgG
6	--	--	--	161	1790
6	Unseparated	1.5	1.5	6	1330
6	Large 20%	0.3	1.5	12	1590
6	Small 75%	1.2	1.5	138	1620
6	Unseparated	0.3	0.3	94	1410
6	Large 20%	0.1	0.5	105	1600
6	Small 75%	0.3	0.4	171	1740

*Indirect DNP-PFC/10^6 recipient spleen cells

TABLE 5

FACS ENRICHMENT OF SUPPRESSOR T CELLS BY SURFACE T DETERMINANTS

SJL x BALB/c Spleen Cells Transferred (x 10^6)			Indirect DNP-PFC*	
	Unprimed Suppressed Nylon T Cells			
Normal DNP 1^0 Spleen	Description	Total Cells	Ig-1b	Total IgG
6	--	--	126	1250
6	Unseparated	1.5	1	1031
6	Dullest 30%	0.75	18	1156
6	Brightest 40%	0.75	98	1000
6	Unseparated	0.3	73	1240
6	Dullest 30%	0.15	90	1235
6	Brightest 40%	0.15	131	1120

*Indirect DNP-PFC/10^6 recipient spleen cells

Splenic T cells (nylon column passed) were stained with a rabbit anti mouse brain serum which was absorbed wtih bone marrow to make it specific for splenic T.

$(Fab')_2$ which was then purified on a G 200 column, fluoresceinated, and used for these studies.) Scatter analysis of the isolated dull fraction shows that it is enriched for large cells and contains a high percentage, although not all, of the large cells in the nylon passed spleen cell suspension.

With these experiments, we have begun the process of isolating suppressor T cells. Hopefully, as the methodology for separating these cells from other functional T cells improves, we will be able to utilize the added resolution obtained by working with purified populations to develop a clear picture of how suppressor T cells prevent Ig-1b memory cells from becoming Ig-1b producing cells.

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