The Journal of Immunology

RESEARCH ARTICLE | JUNE 01 1980

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R C Burton; ... et. al

J Immunol (1980) 124 (6): 2936–2943.
https://doi.org/10.4049/jimmunol.124.6.2936

0022-1767/80/1246-2936$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 124, No. 6, June 1980
Printed in U.S.A.

T CELL RESPONSES TO ALLOANTIGENS

I. Studies of *in Vivo* and *in Vitro* Immunologic Memory and Suppression by Limit Dilution Analysis[1]

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A limit dilution technique was used to study the frequency of cytotoxic T cell precursors (f pre T_c) in the peripheral blood lymphocytes (pbl) of naive and alloimmunized mice. It was found that alloimmune mice showed a 2- to 5-fold specific increase in the f pre T_c reactive to the immunizing alloantigens. This technique was further adapted for use as a sensitive *in vitro* assay for alloantigen-specific suppressor T cells. It was found that nonimmunosuppressed $B6AF_1$ mice bearing surviving B10.BR cardiac allografts had circulating alloantigen-specific suppressor cells. *In vitro* it was shown that the culture of heat-inactivated B10.D2 spleen cells with $B6AF_1$ spleen cells gave rise to alloantigen-specific $B6AF_1$ suppressor cells.

Immunologic responses exhibit as one of their main characteristics the quality of increased and specific responsiveness to repeated stimulation after first contact. This has been termed "memory." The present report describes an approach to the *in vitro* assessment of memory in the cellular compartment of an immune response generated *in vivo*. In particular, a method for the quantification of cells that can be readily converted on additional stimulation to cytotoxic T (thymus-derived) cells (T_C)[3] is described. The system makes use exclusively of cell-surface histocompatibility antigens determined at the major histocompatibility complex (MHC) of genes and assumes that the number of T cells that are capable of responding to a given package of antigens is the critical variable in determining the presence of memory. The results from our study are consistent with this assumption, although it must be recognized that memory may also be reflected in the production of T_C that have a higher affinity for their targets or are capable of reacting with their target cells more effectively for some other reason than would otherwise be the case. Our method has not been designed to reveal these possible elements of a heightened response.

Furthermore, by the use of a very similar application of the dilutional method of analysis of cell reactivity presented here, it has been possible to design a set of circumstances that will reveal cellular suppression of *in vitro* cytotoxicity, and to assay it quantitatively. The following account describes the techniques used, including the problem of data analysis, and presents some examples of how the methods may be applied in measuring the levels of cellular responsiveness of mice repeatedly over a period of time.

MATERIALS AND METHODS

Mice. $B6AF_1/J$, B10.D2/n Sn, and B10.BR/Sg Sn male mice were purchased from The Jackson Laboratories, Bar Harbor, Maine, and used at 6 to 12 weeks of age.

Tissue culture medium (TCM). The medium used in all the studies was RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5×10^{-5} M 2 mercaptoethanol, 50 μg/ml of gentamicin sulfate (Schering Corp., Kenilworth, N. J.), 2 mM glutamine, 1% nonessential amino acids, and 20 mM HEPES buffer (Grand Island Biological Co.). Fetal calf serum (FCS-Grand Island Biological Co.) was added to the medium before use to a final concentration of 10% unless otherwise indicated.

Peripheral blood leukocyte preparations (pbl). Mice were bled from the retro-orbital plexus into heparinized pasteur pipettes, and samples from the same group were pooled. The red cells were lysed by diluting the whole blood 1/10 in isotonic Tris-buffered ammonium chloride and allowing the cell suspension (c.s.) to stand at room temperature for 5 min. The c.s. was then washed three times by centrifugation through a 1-ml FCS underlayer and then resuspended in TCM for a viable cell count by trypan blue exclusion. The usual result was to recover 5 to 10×10^6 viable leukocytes/ml of blood, of which more than 80% were morphologically mononuclear.

Responder spleen cells. Mice were killed by cervical dislocation and their spleens were removed aseptically. A sterile red cell-free single c.s. was then prepared as follows. The spleens were injected with an isotonic solution of Tris-buffered ammonium chloride and then teased apart in the solution. The cells were dispersed by pipetting the solution, and then the c.s. was filtered through a cotton wool-plugged pasteur pipette and made up to 10 ml with TCM. The c.s. was then centrifuged to remove the ammonium chloride, and washed once in TCM before being resuspended for a viable cell count by trypan blue

Received for publication January 21, 1980.
Accepted for publication March 10, 1980.
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[1] This work was supported in part by United States Public Health Service Grants AM-07055 and HL-18646 from The National Institutes of Health.
[2] Dr. Robert C. Burton is a National Health and Medical Research Council of Australia Fellow in Applied Health Sciences.
[3] *Abbreviations used in this paper:* BG, background ^{51}Cr release; c.s., cell suspension; f pre T_c, frequency of cytotoxic T cell precursors; MAX, maximal ^{51}Cr release; MHC, major histocompatibility complex; NHS, normal human serum; pbl, peripheral blood lymphocytes; TCM, tissue culture medium; T_c, cytotoxic T cells; T_s, suppressor T cells; Δ H, heat inactivation.

dye exclusion. Only c.s. of greater than 90% viability were used in the experiments.

Culture conditions. All cultures and assays were performed at 37°C in a humidified incubator in an atmosphere of 5% carbon dioxide in air.

Tumor target cells. Two tumor cell lines, which were maintained in stationary suspension culture (1), were used as targets for alloimmunized Tc in a ^{51}Cr-release assay because they expressed the relevant MHC antigens. They were P815, a DBA/2 (H-2K^d) mast cell tumor, and Cl. 18, a C3H (H-2D^k) plasma cell tumor. Their origins and adaptation to tissue culture have been described previously (2, 3).

These tumor cells were labeled with ^{51}Cr by adding $Na^{51}CrO_4$ (New England Nuclear, Boston, Mass.), 5 μci/ml of TCM to dilute cultures in the logarithmic phase of their growth on the evening before use. The next day the cultures were harvested and washed three times to remove excess ^{51}Cr. This method of ^{51}Cr labeling tumor cell lines usually results in higher ^{51}Cr uptake and lower spontaneous release than pulse labeling (4).

The assay system. The system described herein for detecting Tc memory in animals and suppressor T cells (Ts) is based on the limit dilution technique developed by Lindahl and Wilson (5) for determining the frequency of Tc precursors (f pre Tc) reactive to particular MHC alloantigens in murine spleen cell preparations. The determination of the f pre Tc reactive to H-2K^d and H-2D^k was the first step in the assay described herein.

These f pre Tc were determined by culturing decreasing numbers of viable B6AF$_1$ responder spleen cells or pbl together with 10^6 irradiated (1000 rads) B6AF$_1$ filler spleen cells and 10^6 irradiated B10.D2 or B10.BR stimulator spleen cells in 200 μl of TCM in wells of V bottomed microtiter trays (Linbro Scientific Inc., Hamden, Ct., No. 76-223-05). Groups of 12 to 24 cultures (usually 16) were set up for each responder cell number, and the trays were cultured for 6 days. As the number of responder cells per well is progressively reduced in a stepwise fashion, the proportion of cultures bearing detectable cytotoxic activity in a given group decreases. From this a measure of cell responsiveness to the appropriate antigens can be calculated and changes in this activity over time can be determined.

The cultures were assayed for cytotoxic activity in a ^{51}Cr release assay. This was done by centrifuging the microtiter trays at 300 × G for 3 min and then aspirating all the wells to a common residual volume (100 μl), using an 8-channel vacuum aspirator manufactured in our laboratories. The cell pellets were then resuspended by vibrating the trays on a Micro-Shaker II (Dynatech Laboratories, Alexandria, Va.). Ten thousand ^{51}Cr-labeled target cells (10^5/ml of TCM) were then added to each well with an 8-channel Titertek 100 μl fixed volume Pipette (Flow Laboratories, Rockville, Md.). A set of 12 to 24 cultures was set up for spontaneous (background) ^{51}Cr release (BG) by adding 10^4 ^{51}Cr-labeled target cells to wells containing only TCM. The trays were then centrifuged at 300 × G for 1 min and incubated for 6 hr under the culture conditions described above. At the end of this period 100 μl of the supernatant were removed from each of four wells simultaneously by using a Titertek 4-channel 100 μl fixed volume pipette, deposited in Skatron/Titertek transfer tubes (Flow Laboratories), and then counted in a Beckman Bio-gamma 8000 scintillation counter. Maximal possible ^{51}Cr release value (MAX) was obtained by counting four to eight replicates of 10^4 ^{51}Cr-labeled target cells and halving the mean count. Based on these counts the cultures in each group were then scored positive or negative for cytotoxic activity according to criteria discussed in detail later.

The calculation of the f pre Tc employs a Poisson distribution analysis. The application of this analysis to this end has been dealt with in detail by others (5–8), so only certain aspects of the technique are considered here. When all groups of wells have been scored for negative cultures, there should be a linear relationship between the fraction (percent) of negative cultures plotted on a log scale against the responder number plotted on an arithmetic scale. The f pre T_c is then given by the slope of the line, or the reciprocal of the responder number for 37% negative wells. For comparisons of f pre T_c between different cell suspensions the ratio f_1 pre T_c/f_2 pre T_c is given by

$$\frac{N_2 \text{ (37\% negative wells)}}{N_1 \text{ (37\% negative wells)}}$$

A major consideration in this approach is how the cultures are to be scored positive or negative. The crux of this determination is that the criterion used should clearly distinguish the background release from a positive experimental culture, yet not underestimate the frequency of positive cultures. Other workers have applied themselves to this problem and three methods for scoring cultures have been used:

1) Mean Background (BG) + 4.27 × standard error of the mean (S.E.M.), Lee (7), *designated S.E.*

2) Mean BG + 3 × standard deviation (S.D.), Lindahl and Wilson (5), *designated S.D.*

3) Mean BG + 10% specific lysis, Skinner and Marbrook (8), *designated 10%.*

$$\text{Since \% specific lysis} = \frac{\text{Test count} - \text{BG}}{\text{Max} - \text{BG}} \times 100$$

The figure for 10% is given by: (Max − BG)/10 + BG

An example of the consequences of applying each of the three scoring methods or criteria is illustrated in Table I. Here 2 × 10^4 B6AF$_1$ naive spleen cells have been cultured with 10^6 irradiated B10.D2 spleen cells and 10^6 irradiated B6AF$_1$ spleen cells. The generation of T_c reactive to H-2K^d (B6AF$_1$ anti-B10.D2) has been assayed by using ^{51}Cr P-815 cells. The left

TABLE I

Comparison of analysis of results by three separate criteria

BG[a]	B6AF$_1$[b] Responder 2 × 10^4	Negative Culture
571	726	* #
648	1942	
757	927	#
622	1182	
652	898	#
678	1274	
709	1556	
629	1888	
675	1615	
663	1456	
651	1287	
673	1461	
660 ± 13[c]	Max 3421 ± 147	Criteria[d] S.E. 717 S.D. 798 * 10% 936 #

[a] P-815 target cells only, cpm ^{51}Cr in 100 μl of supernatant.

[b] Counts per minute ^{51}Cr release in individual wells where P-815 target cells were added to a 6-day culture of B6AF$_1$ responder, with B6AF$_1$ filler and B10.D2 stimulator cells.

[c] Mean ± S.E.M.

[d] Criteria of analysis determined from BG and MAX as described in the text. Wells scored negative if cpm was less than a particular criterion, as shown (SE 0/12, SD 1/12, 10% 3/12).

hand column shows the 12 replicate background counts, mean counts per minute (c.p.m.) ± S.E.M. (600 ± 13). In the right hand column the 12 test counts have been independently scored as "negative" according to each of the three criteria: 0/12 for S.E., 1/12 for S.D. (*), and 3/12 for 10% (#). All three criteria clearly distinguished positive cultures from background release. The most "sensitive" criterion (S.E.) was most susceptible to background variation.

In order to compare the utility of all three criteria of analysis, cultures were scored by all of them in this paper. The choice of the criterion to be applied in a particular experiment has been based upon two considerations. 1) The requirements detailed above, i.e., to detect all positive cultures and yet clearly distinguish background release, and 2) the needs of the particular experiment, i.e., the detection of memory *versus* suppression. Because of the nature of the measurements available in this system it must be accepted that any cutoff point for scoring cultures as negative must be arbitrary. As long as the distinction employed delineates test from background results clearly, is applied to *all* groups within a given experiment, and results in a linear plot as discussed above then it can be considered to yield valid comparisons. Therefore, experiments employing this analysis must be internally controlled, and comparisons between experiments must be based on the use of consistent internal controls. These points will be illustrated below.

Once the f pre Tc has been determined for naive spleen cells or pbl reactive to MHC alloantigens of another strain, sensitive assays for Tc "memory" and Ts can be performed in that strain combination.

Assay for Tc memory. $B6AF_1$ mice were alloimmunized to B10.D2 ($H\text{-}2K^d$) or B10.BR ($H\text{-}2D^k$) alloantigens by skin grafting, as described by Billingham and Medawar (9). Groups of four to eight $B6AF_1$ mice received either B10.D2 or B10.BR skin grafts and then were tested for T_c memory at various times up to 6 months after the grafts were rejected. The presence of T_c memory was determined in the first instance by measuring the f pre T_c reactive to the donor alloantigens and to 3rd party alloantigens in the pbl of immunized and naive $B6AF_1$ mice. This approach was subsequently simplified by reducing the number of groups of cultures and the range of responder cell numbers used.

Assay for Ts. This approach was then employed to develop a system that could be used both as a sensitive detection assay for Ts, and also as an assay of the amount (strength) of suppression.

1) Sensitive Ts assay. A responder spleen cell number was chosen that, together with filler and stimulator cells, would give 10 to 50% negative cultures for a particular strain combination in the assay system for f pre Tc described above. Cell suspensions containing putative Ts were set up in groups of 12 to 24 replicates in wells of V bottom microtiter trays over a range of cell numbers in 100 μl of TCM. Then responder, filler, and stimulator cells were added to all the wells in 100 μl of TCM. An additional group of 12 to 24 control wells that had not received Ts was also included. The rest of the assay was then as described above. Suppression was detected by a significant increase in the percentage of negative cultures over that of the control (10 to 50% negative). The specificity of suppression was tested for by adding cells from strain combinations of responder, filler, and stimulator to which specifically active suppressor cells should not have been generated. The cell class of the suppressor cells was determined by treating aliquots of the cell suspensions with rabbit complement, (C) or anti-Thy 1.2 serum and C, as described elsewhere (10), and then assaying them as described above.

2) Insensitive Ts assay. In order to gain an estimate of the "strength" of suppression the assay described above was set up using a responder cell number that was calculated to give less than 10% negative cultures in the absence of suppression.

Production of suppressor cells. Two sources of putative alloantigen specific Ts were utilized for the purpose of developing the assay described above. *a) In vivo*: When B10.D2 or B10.BR kidneys are transplanted orthotopically to bilaterally nephrectomized $B6AF_1$ recipients, or a B10.BR heart is transplanted heterotopically into the abdomen of a $B6AF_1$ recipient by primary vascular union, the majority of the grafts survive and function indefinitely without any immunosuppression (11, 12). Furthermore, although B10.D2 or B10.BR skin grafts are rapidly rejected by normal $B6AF_1$ mice, surviving $B6AF_1$ recipients of B10.D2 kidneys reject B10.D2 skin grafts much more slowly if at all, although third party skin is rejected promptly (11). Similar findings also apply to $B6AF_1$ mice bearing long term functioning B10.BR cardiac allografts (13). Since these *in vivo* observations suggested that the recipients of primarily vascularized organ grafts developed an active alloantigen-specific unresponsiveness, pbl from these animals were assayed for the presence of T_s. *b) In vitro*: We have recently found that suppressor cells can be induced *in vitro* by culturing viable murine spleen cells with heat-inactivated allogeneic spleen cells.[4] $B6AF_1$ spleen cells (20×10^6) were cultured together with 20×10^6 heat-inactivated (Δ H-45°C for 60 min) B10.D2 spleen cells in Falcon flasks (Falcon Plastics, No. 3012) in 10 ml of TCM supplemented with normal human serum (NHS, Bio Bee Laboratories, Boston, Mass.). The culture conditions were as described above, and after 6 days the cells were harvested, a viable cell count was performed, and then the cells were assayed for suppressive activity.

RESULTS

Determination of the f pre Tc and need for filler cells. The f pre Tc reactive to B10.D2 alloantigens (anti-K^d) in $B6AF_1$ spleen cells and pbl were determined as follows. Cultures containing cell numbers ranging from 10^3 to 2×10^4 $B6AF_1$ responder spleen cells or 5×10^3 to 5×10^4 $B6AF_1$ responder pbl obtained from the same animals were set up together with 10^6 irradiated filler $B6AF_1$ and 10^6 irradiated stimulator B10.D2 spleen cells in groups of 16 micro wells for each responder cell number. The wells were assayed 6 days later with ^{51}Cr P-815 ($H\text{-}2K^d$) and Figure 1 shows the results from a representative experiment. Here the data, analyzed according to each of the three criteria of analysis (S.E., S.D., and 10%), are shown for the $B6AF_1$ spleen cell responders, and are compared with the S.E. plot of the $B6AF_1$ pbl responders. It can be seen that the criterion used to score the wells can significantly affect estimates of the f pre T_c for $B6AF_1$ spleen: 1:5800, SE: 1:8500, SD: 1:11,600, 10%. However, since the assay was being used here for comparative purposes an accurate determination of the absolute f pre Tc was not required. By these determinations it can be seen that there is at least a 3-fold higher frequency of $B6AF_1$ pre T_c reactive to B10.D2 alloantigens in the spleen as compared to pbl (S.E. spleen 1:5800, SE pbl 1:18,400). In all subsequent experiments cultures were scored according to all three criteria, and the criterion that yielded the most decisive result was chosen, i.e., at least 3 points on a linear plot over a wide range. This is indicated in the text for each experiment.

A second similar experiment is shown in Figure 2. Here the

[4] Burton, R. C., C. F. Shield, A. B. Cosimi, and P. S. Russell. *In vitro* induction of human and murine alloantigen specific suppressor T cells. Submitted for publication.

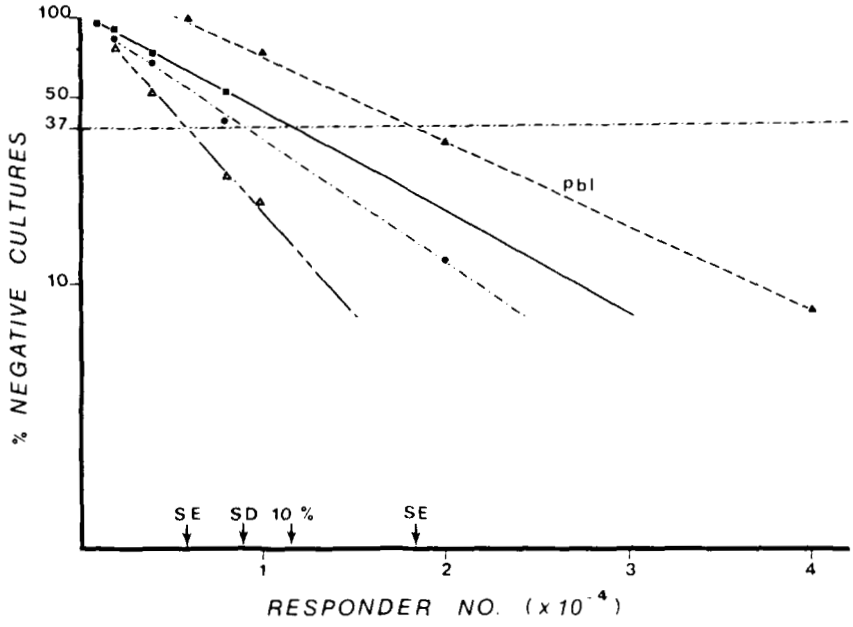

Figure 1. Criteria of analysis of f pre T_c for $B6AF_1$ naive spleen cells or pbl reactive to B10.D2 alloantigens (anti H-$2K^d$) assayed with ^{51}Cr P-815. Spleen cells, S. E. (△), S.D. (●), 10% (■), and pbl SE (▲). f pre T_c given by 37% negative wells (zero order Poisson distribution analysis).

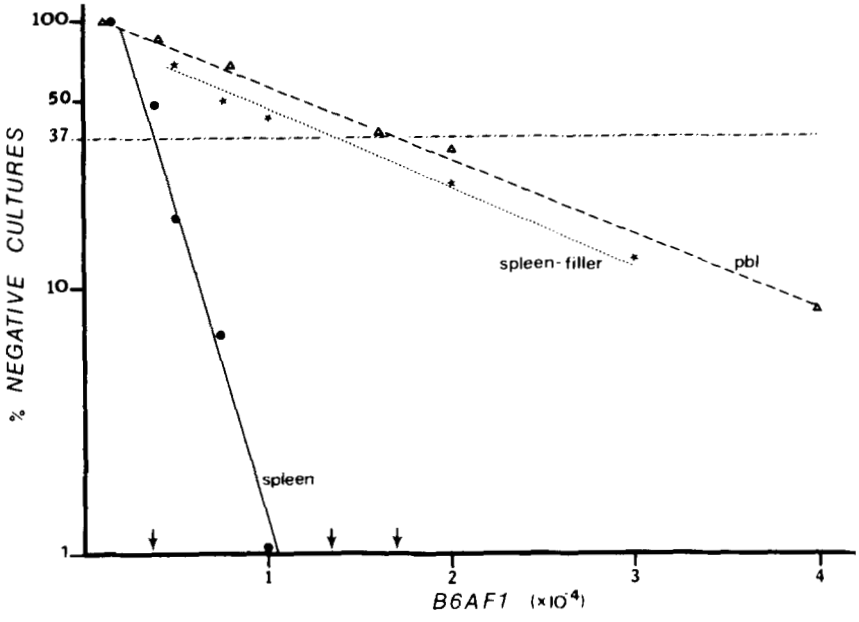

Figure 2. Role for filler cells in limit dilution assay. $B6AF_1$ spleen cells responding to B10.D2 alloantigens with filler cells (●) or without (★) compared to $B6AF_1$ pbl responding with filler cells (△). Analysis by S.E. criterion, f pre T_c as 37% negative cultures.

results are plotted for the S.E. criterion, and the f pre T_c reactive to B10.D2 alloantigens in spleen was 5-fold higher than in the pbl. In this experiment a second identical group of responder spleen cell cultures was set up without the filler spleen cells. The results are in agreement with those of others that show that a radio resistant cell MHC compatible with the responder is essential in this assay (14).

Detection of Tc memory in alloimmunized mice. The f pre T_c in the pbl of $B6AF_1$ mice that had rejected either a B10.D2 or B10.BR skin graft 1 to 2 months before testing was compared to that of naive age- and sex-matched $B6AF_1$ control animals. The results are illustrated in Figures 3 and 4, in which it can be seen that the immune animals had a higher frequency of T_c precursors reactive to the immunizing alloantigens. In the case of the $B6AF_1$ pbl response to B10.D2 cells (Fig. 3) assayed with ^{15}Cr P 815 (H-$2K^d$), the f pre T_c was 2-fold higher in the immune animals when analyzed by using the S.E. criterion. For the $B6AF_1$ mice that had rejected a B10.BR skin graft (Fig. 4) the f pre T_c reactive to H-$2D^k$ in the pbl assayed with ^{51}Cr CL.18 (H-$2D^k$) was 5-fold higher than in naive control mice. Therefore $B6AF_1$ mice that had rejected either B10.D2 or B10.BR skin grafts had a higher f pre T_c in pbl reactive to the relevant alloantigens than naive mice.

These T_c precursors in the immune mice were specific for the immunizing alloantigens. In Table II is shown the results from two of a series of experiments in which three groups of eight age- and sex-matched $B6AF_1$ animals were followed for T_c memory in pbl. The members of one group had received B10.D2 skin grafts; a second group received B10.BR skin grafts, whereas the third remained ungrafted (naive controls). Each group of mice was pool bled as described, and groups of 16 identical cultures were set up with responder pbl numbers, which for the naive mice would give 50 to 75% negative cultures for the particular stimulator cells. Thus, it can be seen that 10^4 naive $B6AF_1$ responder pbl cultured with 10^6 irradiated $B6AF_1$ filler and B10.D2 stimulator spleen cells gave 50% negative cultures (8/16) when assayed with ^{51}Cr P-815, as analyzed by the S.E. criterion. However, 10^4 pbl from $B6AF_1$ mice that had rejected

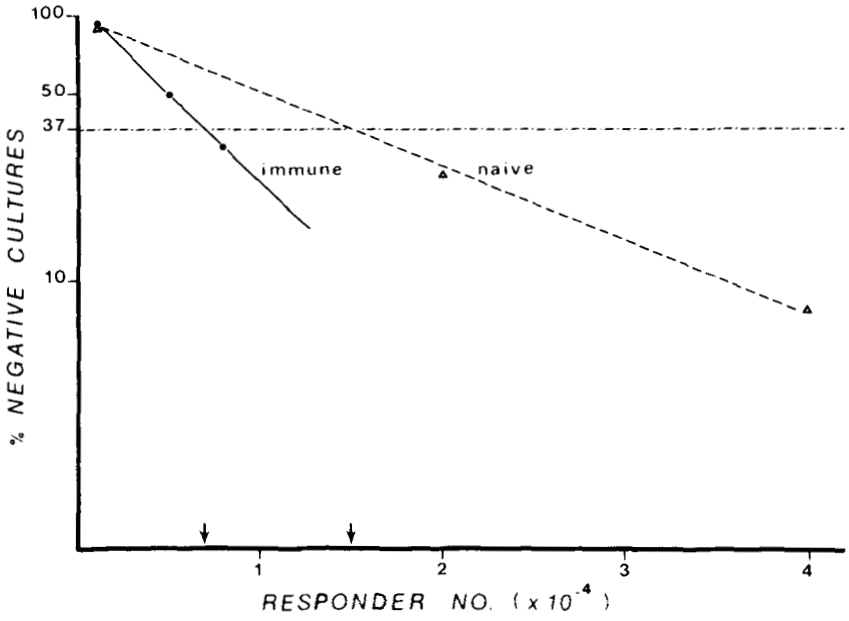

Figure 3. Detection of memory to H-2K^d in pbl. Naive (△) or immune (●) B6AF$_1$ pbl respond to B10.D2 alloantigens. Analysis by S.E. criterion.

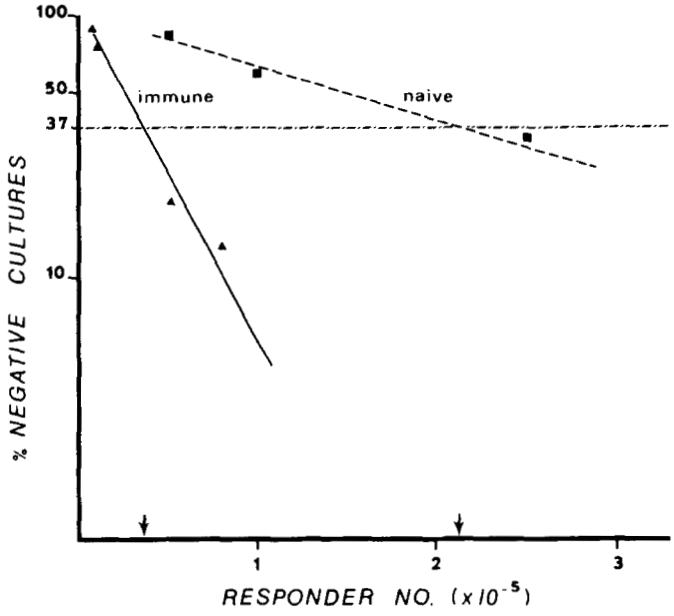

Figure 4. Detection of memory to H-2D^k in pbl. Naive (■) or immune (▲) B6AF$_1$ pbl respond to B10.BR alloantigens. Analysis by S.E. criterion.

a B10.D2 skin graft gave only 6% (1/16) negative cultures when cultured and assayed under the same conditions. By contrast pbl from B6AF$_1$ mice that had rejected a B10.BR skin graft gave more negative cultures (81%) than the controls. The specificity of T$_c$ memory assayed in this way was confirmed by stimulating 10^5 pbl from the same pools with B10.BR cells (Table II).

The data above have been presented in this single point fashion rather than as absolute f pre T$_c$ because the results were taken from an experiment in which these three groups of mice were followed for 10 months after sensitization. The mice were eyebled every 2 to 4 weeks, and the pooled pbl from each group tested for memory and specificity in this fashion. Although the limit dilution analysis was performed in full once, it was found to have no advantage over this simple method that required less blood.

Assay of Ts induced in vitro. B6AF$_1$ viable spleen cells were cultured together with heat inactivated (Δ H) B10.D2 spleen cells for 3 to 6 days. The viable cells were then harvested from these cultures and assayed by adding them to groups of 12 fresh cultures of B6AF$_1$ responder, B6AF$_1$ filler and B10.D2 or B10.BR stimulator cells. In the experiment shown (Table III) B6AF$_1$ spleen cells were cultured with Δ H B10.D2 spleen cells for 6 days (Test 1 results) or 3 days (Test 2 results) and then tested for suppression by adding 3×10^3 viable cells to groups of 12 wells containing 2×10^4 B6AF$_1$ normal responder, 10^6 irradiated B6AF$_1$ filler and 10^6 B10.D2 irradiated stimulator spleen cells. The results shown in Table I were also derived from this experiment. The S.E. criterion was not used in the analysis of suppression since all the control cultures (no suppressors) were positive. When the control, Test 1, and Test 2 groups of 12 were scored by the S.D. criterion (*798) or 10% criterion (≠936) it can be seen that the Test 1 cells suppressed the induction of T$_c$ from the pre T$_c$, but the Test 2 did not. That is to say, 3 of 12 control, 9 of 12 Test 1, and 3 of 12 Test 2 cultures were negative by the 10% (≠) criterion.

Table IV shows the results of two experiments, where B6AF$_1$ spleen cells that had been cultured with Δ H B10.D2 spleen cells for 6 days were assayed for alloantigen-specific suppression. In the first, analyzed by the 10% criterion, the assay was set up for sensitivity with 20% negative cultures in the B10.D2 stimulator control (nil suppressor) group and 30% in the B10.BR stimulator control group. When 10^4 or 10^3 putative suppressor cells were added to these cultures alloantigen-specific suppression was demonstrated, i.e., 10^4 B6AF$_1$ anti-ΔH B10.D2 cells *increased* the percentage of negative cultures from 20 to 83% in the specific system (B6AF$_1$ responder, B10.D2 stimulator), whereas in the specificity control (B6AF$_1$ responder, B10.BR stimulator) the same cells in fact *reduced* the percentage of negative cultures from 30 to 8, probably because these B6AF$_1$ cells acted as additional responder cells.

In the second experiment analyzed by the S.E. criterion the control cultures in the specific and control systems both yielded only 6% negative cultures, so the assay was relatively insensitive. Putative B6AF$_1$ anti-AH B10.D2 suppressor cells added to these systems produced specific suppression, and in this experiment 3×10^3 suppressor cells increased the percentage of negative cultures in the specific system to 69%. There was no suppression detectable in the control system. When the B6AF$_1$

TABLE II

In vitro specificity of T_C memory to alloantigens in mice immunized by skin grafting

Responder pbl	Stimulator Spleen Cells (1000 rads)	Filler Spleen Cells (1000 rads)	% Negative Cultures[a]
10^4	10^6	10^6	^{51}Cr *P-815*
Naive $B6AF_1$	B10.D2	$B6AF_1$	50
$B6AF_1$/B10.D2 S.G.[b]	B10.D2	$B6AF_1$	6
$B6AF_1$/B10.BR S.G.	B10.D2	$B6AF_1$	81
10^5	10^6	10^6	^{51}Cr *C1.18*
Naive $B6AF_1$	B10.BR	$B6AF_1$	75
$B6AF_1$/B10.D2 S.G.	B10.BR	$B6AF_1$	86
$B6AF_1$/B10.BR S.G.	B10.BR	$B6AF_1$	6

[a] $B6AF_1$ anti-B10.D2 assayed for anti-H-$2K^d$ with ^{51}Cr P-815 (H-$2K^d$). $B6AF_1$ anti-B10.BR assayed for anti-H-$2D^k$ with ^{51}Cr C1.18 (H-$2D^k$). Analysis by S.E. criterion.

[b] $B6AF_1$ mice had been grafted with B10.D2 or B10.BR skin 22 days previously. Rejection of the grafts had been completed 7 to 9 days before testing.

TABLE III

Suppressor cell assay analysis

BG[a]	Control[b] 2×10^4	Test 1[c] 5×10^3	Test 2[d] 5×10^3
571	726 * #	812 #	626 * #
648	1942	1020	811 #
757	927 #	639 * #	1628
622	1182	740 * #	1361
652	898 #	870 #	938
678	1274	773 * #	754 * #
709	1556	685 * #	1134
629	1888	683 * #	1133
675	1615	783 * #	943
663	1456	933 #	1761
651	1287	1093	1773
673	1461	980	1100
660 ± 13	MAX 3421 ± 147	Criteria[e]	S.E. 717 S.D. 798 * 10% 936 #

[a] P-815 target cells only.

[b] Responder, filler, and stimulator cells + nil suppressor controls.

[c] Putative suppressor cells added from a 6-day culture of $B6AF_1$ responder and Δ H B10.D2 stimulator cells.

[d] Putative suppressor cells added from a 3-day culture of $B6AF_1$ responder and Δ H B10.D2 stimulator cells.

[e] All results expressed in counts per minute for individual wells, analysis as for Table 1.

anti-Δ H B10.D2 cells were pretreated with anti-Thy 1.2 serum and C specific suppression was abolished,[4] indicating that the alloantigen-specific suppression was mediated by T cells.

Detection of suppressor cells in mice bearing surviving organ transplants. Healthy nonimmunosuppressed $B6AF_1$ mice bearing primarily vascularized heterotopic B10.BR heart allografts for periods of 1 to 3 months were tested for the presence of suppressor cells. The technique used was as described for the assay of T_s induced *in vitro*, except that the putative suppressor cells were pbl from these allografted mice. In a number of experiments definite evidence for the presence of suppressor cells was found. The results of a representative experiment illustrating this finding are shown in Table V. Here, $B6AF_1$ responder cell numbers of 10^5 and 3×10^4 were used, which resulted in 25% negative cultures (4 of 16) in the nil suppressor controls when the wells were scored by the 10% criterion. When 2×10^5 pbl from $B6AF_1$ mice bearing B10.BR heart allografts were added at the initiation of culture, 75% of the $B6AF_1$ anti-B10.BR cultures were negative. There was no suppression detected in the specificity control ($B6AF_1$ anti-B10.D2). This technique has also been successfully used to detect alloantigen-specific suppressor cells in the blood of $B6AF_1$ mice bearing B10.D2 orthotopic primarily-vascularized renal allografts.

With numerous repetitions of this experiment, in which suppressor cells were sought for in the blood of these animals, their presence could not be determined on a regular basis. Thus, in 20 trials significant alloantigen-specific suppression was detected in 11, and no definite trend was noted in the prevalence of detection of suppressor cells as related to the time that had elapsed after transplantation. In order to exclude technical failure as an explanation for the negative results, suppressor cells generated *in vitro*, as described earlier, were used as positive controls in a number of assays. These controls always showed alloantigen-specific suppression, whereas pbl from transplanted animals gave both positive and negative results in the same assays (data not shown).

DISCUSSION

This report describes not only techniques that have been developed for the detection and enumeration of T cell memory and suppression, but also includes examples of circumstances in which these techniques were employed to evaluate cell populations derived directly from animals. Thus, the rejection of a skin graft has been demonstrated to leave the recipient with a readily detectable increase in the f pre T_c in its peripheral blood. These cells respond *specifically* to further stimulation by appropriate donor antigens and not to those characteristic of a third party. In our experiments (over 40 experiments) in the strains described herein the f pre T_c in the pbl of alloimmunized animals has ranged from 2- to 6-fold that of control naive animals. It has *never* been less. We have found that this

TABLE IV

Specificity of suppressor cells induced in NHS

Responder Cells	Stimulator Cells	Filler Cells	Suppressor Cells	% Negative Cultures[a]
$B6AF_1$	B10.D2	$B6AF_1$		^{51}Cr *P-815 (H-$2K^d$)*
5×10^4	10^6	10^6	Nil	20[b]
			$B6AF_1$ anti-B10.D2	
			10^4	83
			10^3	33
$B6AF_1$	B10.BR	$B6AF_1$		^{51}Cr *C1.18 (H-$2D^k$)*
10^5	10^6	10^6	Nil	30[b]
			10^4	8
			10^3	33
$B6AF_1$	B10.D2	$B6AF_1$		^{51}Cr *P-815*
2×10^4	10^6	10^6	Nil	6[c]
			$B6AF_1$ anti-B10.D2	
			10^4	88
			3×10^3	69
$B6AF_1$	B10.BR	$B6AF_1$		^{51}Cr *C1.18*
10^5	10^6	10^6	Nil	6[c]
			10^4	6
			3×10^3	6

[a] Target cells P-815 or C1.18 as shown.

[b] Analysis by 10% criterion.

[c] Analysis by S.E. criterion.

TABLE V

Alloantigen specific suppressor cells detected in cardiac allograft recipients

Responder Cells	Stimulator Cells	Filler Cells	Suppressor Cells	% Negative Cultures[a]
$B6AF_1$	B10.BR	$B6AF_1$		^{51}Cr *C1.18*
10^5	10^6	10^6	Nil	25
			pbl B6AF$_1$ B10.BR heart	
			2×10^5	75
$B6AF_1$	B10.D2	$B6AF_1$	Nil	^{51}Cr *P-815*
3×10^4	10^6	10^6	*pbl B6AF$_1$ B10.BR heart*	25
			2×10^5	25

[a] Analysis by 10% criterion.

increase in the f pre T_c in the pbl after rejection of a skin allograft is not necessarily permanent, and that the f pre T_c returns to normal over a period of some months when certain strain combinations are used. (Burton and Russell, manuscript in preparation.) Since this increase in the f pre T_c is specific for the immunizing alloantigens, it may constitute an essential quantitative aspect of T_c memory. Ryser and Macdonald (15) have recently reported similar findings for the f pre T_c in spleen cell suspensions from mice immunized by a single i.p. injection of allogeneic tumor cells. Further studies would be necessary, however, to prove that it is essential for T_c memory for an increase in f pre T_c to occur, since it is possible that pre T_c from alloimmunized animals are simply more readily inducible *in vitro* (16), and/or kill more efficiently in the ^{51}Cr release assay (17). Properties such as these could create "positive" cultures in a detection system whose sensitivity is still not certain. This technique, however, offers an opportunity for the study of specific T_c reactivity to alloantigens in groups of mice or even in individual mice over prolonged periods.

$B6AF_1$ mice that have received B10.BR heterotopic, abdominal, primarily vascularized heart transplants usually manifest a phase of active rejection activity between the 3rd and 5th weeks after transplantation, which is associated with a marked decrease in the vigor of the palpable cardiac pulsation (11). Later the graft may be restored gradually toward normal, with a much more active pulse being palpable for an extended period thereafter (13). Accordingly animals bearing such allogeneic transplants were felt to be appropriate subjects in which to search for cells with suppressor activity. Our results have shown that such cells can indeed be detected by the method described. Similar results have been obtained in mice bearing orthotopic primarily vascularized renal allografts. The unresponsiveness to primarily vascularized allografts that occurs in nonimmunosuppressed mice in certain strain combinations has been described in previous communications from this laboratory (12, 13). The present finding of alloantigen-specific suppressor cells in the blood of such animals is new. Furthermore, treatment with anti-Thy 1.2 serum and C abolished the suppressive effect of these cells (unpublished observations), suggesting that they were T_s.

These observations might suggest that since T_s are induced by the introduction of a primarily vascularized allograft, these same T_s subsequently maintain unresponsiveness. However, two additional observations render this an uncertain conclusion. We have to date been unable to transfer adoptively this state of unresponsiveness, despite the use of heavily irradiated or anti-thymocyte globulin-treated recipients and the transfer of 1 to 2×10^8 spleen cells. Furthermore, the *in vitro* detection of T_s by the method described shows marked variability. When individual and groups of mice were tested repeatedly over many months T_s were detected only intermittently. The concurrent use of T_s induced *in vitro* as a positive control, however, does enable us to affirm that T_s are indeed present in the pbl, at least from time to time. Similar findings have been reported by Gorczynski and McRae (18), who found that T_s capable of suppressing the induction of T_s from pre T_s *in vitro* were detectable in only 40 to 50% of the spleens of tolerant mice, 45 days after neonatal injection of allogeneic lymphoid cells. These authors also reported data that suggested that the presence of a second distinct T_s type in tolerant animals seemed to restrain the production of pre T_c from stem cells. Furthermore, they also suggested that tolerance induction might be a function of the former T_s type and maintenance of tolerance a function of the latter T_s type. The experimental technique reported here would detect only those T_s that control the differentiation of T_c from their precursors. T_s that act at a prethymic stage (18), or a third possible T_s type that would prevent the expression or recall of T_s memory would not be detected.

We are currently exploring the *in vivo* role of alloantigen-specific T_s in the induction and maintenance of tolerance of both free and primarily vascularized allografts in mice, using T_s induced *in vitro* and *in vivo*. In particular we are endeavoring to evaluate the significance of the presence of T_s by *in vitro* assays. Perhaps the suppressor T cell is not the sole, or even the most important mediator of specific unresponsiveness to allografts *in vivo* in the systems that we have been studying (19).

Acknowledgments. The authors wish to acknowledge the excellent technical assistance of Ms. Renee Aversano and the contribution of Ms. Catharine Chase.

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