

P815 TUMOR-SPECIFIC T SUPPRESSOR  
CELL AND SUPPRESSOR FACTOR

by

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## ABSTRACT

The work reported here involves studies of suppressor T cells ( $T_sC$ ) and their suppressor factor (SF) which specifically suppress the in vitro generation of cells cytotoxic for a syngeneic tumor, P815, in DBA/2 mice. This work can be divided into three sections: a) the immunogenetic properties and requirements of this  $T_sC$  and SF, b) the Lyt phenotype of the  $T_sC$  as well as that of the cells involved in the cytotoxic response to the syngeneic tumor, c) the properties of syngeneic and allogeneic antisera raised to the P815 specific SF.

a) P815-antigen specific  $T_sC$  and suppressive extracts obtained from the thymuses of DBA/2 mice bearing small syngeneic P815 tumors, were compared for their immunogenetic properties and requirements. It was shown that pretreatment of  $T_sC$  populations with anti-Ia<sup>d</sup> antiserum plus rabbit complement removed the suppressive activity. Similarly, absorption of the SF with anti-Ia<sup>d</sup> antiserum removed the suppressive properties of the material. It was found that the  $T_sC$  and SF were capable of specifically suppressing the anti-P815 response of B6D2F<sub>1</sub> radiation chimeras possessing lymphoid cells of the H-2<sup>b</sup> or H-2<sup>t2</sup> haplotype equally as well as they could suppress the response of H-2<sup>d</sup> bearing cells. This indicates that the  $T_sC$  and SF are not H-2 restricted with respect to K or D markers on responder cells in this system.

b)  $T_sC$  were also identified in the spleens of DBA/2 mice injected intraperitoneally with membrane extracts of the P815 tumor. The Lyt

phenotypes of various effector cells was determined. DBA/2 allogeneic killer cells were identified as  $\text{Lyt-1}^{+}\text{2}^{+}$ , whereas the syngeneic effector cells were found to be predominantly  $\text{Lyt-1}^{-}\text{2}^{+}$ . The suppressor cell population lost its ability to suppress the in vitro cytotoxic anti-P815 response after treatment with anti-Lyt-1 serum plus complement but not after treatment with anti-Lyt-2 serum, indicating that an  $\text{Lyt-1}^{+}\text{2}^{-}$  cell is essential in this suppression.

c) P815 tumor-specific SF was partially purified by passage of suppressive spleen extracts through an immunoadsorbent containing P815 membrane components. Antisera raised in syngeneic DBA/2 and allogeneic, C57BL/6, mice were tested. It was found that these antisera, but not their controls were capable of absorbing out the SF. The antisera were also capable, in the presence of complement, of eliminating  $\text{T}_\text{S}$ C from suppressive spleen cell populations. However, the antisera were not capable of eliminating syngeneic tumor specific in vitro generated killer cells, indicating that the receptor molecules on suppressor and effector cells in this system are distinct from each other. Only the antisera raised in syngeneic DBA/2 mice had any observable effect on P815 tumor growth in vivo.

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FOR DEBBI

CHAPTER I  
INTRODUCTION

## CHAPTER I

Introduction

It is now apparent that the immune response is the result of a complex series of ongoing interconnecting events which is only initiated by antigenic stimulation (1). Initially, the immune response was thought of as a physiological response of the body to antigen, in which the sole end product was antibody. Later, the two main limbs of the immune response were recognized (humoral and cell-mediated), and within the past two decades, the two lymphocyte subpopulations mediating these responses were defined. Thus it was realized that at least two types of interactions occurred between antigen and the two responding cell types, the T and B lymphocytes. This was followed by the realization that the immune response is regulated via T cell-B cell interactions, and also T cell-macrophage, and T cell-T cell interactions. It is now recognized that the T lymphocyte population is made up of distinct subsets which interact with each other as well as B cells and macrophages, to both amplify and inhibit a variety of immunological responses. T cells have demonstrated enhancing effects on the immune response by way of helper T cells ( $T_H$ ) (2-10). T cells have also been found to inhibit the immune response by way of suppressor T cells ( $T_S$ ) (11-44). Although some of these studies have demonstrated the existence of non-specific regulatory T cells, the antigen specific regulatory T cells are especially intriguing because they appear to possess the specificity that usually marks the immune response.

One of the first observations of antigen-specific  $T_s$  were made regarding antibody formation in rats (45-47). This was quickly expanded to include several different antigenic systems (11-17,22-44, 48-58). From this and other work some general characteristics of antigen-specific  $T_s$  begin to emerge. First they show specific antigen or idiotype binding potential. Antigen specific  $T_s$  also express Ia determinants. This is usually I-J coded. The inhibitory activity of these cells may or may not be genetically restricted to self H-2 type. The antigen-specific  $T_s$  in many experimental systems have a surface phenotype of  $Lyt-1^{-}2^{+}$ . However, there are also some reports of  $T_s$  having the  $Lyt-1^{+}2^{-}$  phenotype or even  $Lyt-1^{+}2^{+}$ .

In many of the investigations concerning antigen-specific regulatory T cells, it has been shown that soluble factors derived from these cells can apparently duplicate their function (59-61). Thus, the antigen-specific factors once produced may work independently of the T cells which produced them. This would have the effect of increasing the effective radius of the regulatory T cell, much in the same way that antibody functions with respect to the B cell. These factors may also interact via network type interactions to induce the activation of other lymphocytes, which eventually leads to the final net effect on the immune response. This has been best studied in the antigen-specific suppression systems (60).

As in the antigen-specific  $T_s$  investigations, the initial discovery of antigen-specific suppressor factor (SF) was made by Tada and colleagues (62-64). Again, this observation was quickly broadened to many other experimental systems (47,59-61,65-80). The antigen-specific

suppressor factors exhibit several physical and functional characteristics in common. They are produced or extracted from antigen primed  $T_s$ . They are proteins with specific antigen (or idiotype) binding potential. Their molecular weight is usually between 35-75,000. They lack constant region immunoglobulin determinants but may carry variable (idiotypic) determinants of the immunoglobulin heavy chain. They have determinants which are encoded by the Ir region (usually I-J), and like the antigen-specific  $T_s$  they may or may not be genetically restricted in their effect to acting on cells of the same H-2 type. Finally, since T cells would be unlikely to express antigen-specific molecules unless they were receptors, antigen-specific suppressor factors are an obvious candidate for the antigen receptor of  $T_s$ . Several reviews have been published recently on antigen-specific T cell factors, and one is directed there for a more comprehensive examination of antigen-specific suppressor factors (59-61).

The early investigations carried out by Takei and colleagues in this laboratory provided preliminary data on which the work in this thesis is based. They found that DBA/2 mice injected with syngeneic P815 mastocytoma cells 8 days previously, contained in their thymus P815-specific  $T_s$  (P815- $T_s$ ), which could inhibit the in vitro generation of P815-specific cytotoxic T cells (P815- $T_c$ ) (11,12). The experimental design for demonstrating this was as follows. The in vitro assay for cytotoxic T cells involved the culturing of "immune" DBA/2 splenocytes (taken 12 days following subcutaneous injection of a small number of P815 cells into the DBA/2 mice) with mitomycin-C treated P815 cells for 4 days prior to cell harvesting. Specific cytotoxicity was measured by a

standard 18 h  $^{51}\text{Cr}$  release assay. When thymocytes from DBA/2 mice, inoculated 8 days previously with P815, were cocultured in this system, the generation of cytotoxic T cells was greatly reduced as assessed by the reduction in  $^{51}\text{Cr}$  release mediated by these cultures in comparison to appropriate controls. This suppressive effect was shown to be antigen-specific.

It was later shown that a P815-specific suppressor factor (P815-SF) could be isolated from sonicated P815- $\text{T}_\text{s}$  extract (65). This SF could replace the P815- $\text{T}_\text{s}$  in the in vitro functional assay. The P815-SF was capable of suppressing the in vitro generation of syngeneic cells cytotoxic for P815 tumor cells if it was added during the first 30 h of culture. It was found to have a molecular weight in the range of 40-60,000, with an isoelectric point in the range of 4.6-4.9. The P815-SF could be removed by passage through immunoadsorbent columns prepared from membrane fragments of P815 but not by analogous columns prepared with L1210 (syngeneic leukemia of DBA/2) membrane fragments. The P815-SF was also not removed by passage through immunoadsorbent columns containing anti-mouse immunoglobulin antibody.

The work reported here involved continued studies of these suppressor T cells and the suppressor factor extracted from them. Both the cell and factor are P815 specific and obtained from DBA/2 mice primed with P815 tumor cells or P815 membrane fragments. The functional assay involves the ability of either cells or their extracts to specifically inhibit a primary in vitro cytotoxic response of normal DBA/2 splenocytes to P815 tumor cells. The work in this report has been divided into three self-contained chapters for ease of reading.

Chapter II examines some of the immunogenetic properties and requirements of the P815-T<sub>s</sub>. This work was carried out between 6/77 and 9/78. Chapter III examines the Lyt phenotype of the P815-T<sub>s</sub> as well as that of the cells involved in the cytotoxic response to the syngeneic P815 tumor. This investigation was carried out between 6/78 and 2/79. The work reported in Chapter IV examines the in vitro and in vivo effects of syngeneic DBA/2 and allogeneic C57BL/6 antisera raised to P815-SF. This work was done between 10/78 and 5/81. Chapter V is a summary discussion of the studies reported in the preceeding three chapters.



## CHAPTER II

### CHARACTERIZATION OF THE H-2 PROPERTIES AND REQUIREMENTS OF P815 TUMOR-SPECIFIC SUPPRESSOR T CELLS AND THEIR SOLUBLE FACTOR

## CHAPTER II

Introduction

There have been a number of reports showing that the lymphoid organs of tumor-bearing mice contain T cells that are capable of specifically suppressing the response of normal cells to that tumor (12,15,36,83-85). It has also been shown that, in some cases, it is possible to isolate from such populations of suppressor cells, soluble factor(s) also capable of inhibiting the specific anti-tumor response of normal lymphoid cells (65,76,86,87).

Studies on the characterization of soluble specific suppressor factors in tumor-bearing (65,76,86) animals have shown that they do not appear to differ significantly from antigen-specific suppressive factors demonstrated in animals immunized with a variety of antigenic stimuli (59-61,77,81,82,88). Generally, suppressive factors have been found to have m.w. in the range of 30-75,000. Moreover, they have been shown to exhibit antigen specificity (65,75-78,87) and not to share antigenic similarities with the constant regions of immunoglobulin (65,77,78,87). Studies with these factors have also shown that they can be removed by absorption with antisera raised to products of the major histocompatibility complex (MHC) excluding the K and the D regions (anti-Ia serum). Further analyses of these absorptions have shown that the subregion of the Ia that is expressed on the suppressor factor is encoded by the I-J region (77,81,82,86). A recent observation that treatment of tumor bearing animals with small amounts of anti-I-J antiserum caused a significant slowing in the growth of syngeneic tumor (89) indicate that elimination of I-J-

bearing cells in vivo may be of a clinical significance.

The present study was undertaken to determine whether suppressor cells elicited in tumor-bearing animals were capable of suppressing the primary in vitro cytotoxic response of normal cells to tumor antigens, and to compare the suppressor cells, thus generated, with the equivalent soluble factors in terms of Ia-bearing characteristics and genetic restrictions regarding their suppressive qualities.

## Materials and Methods

### Mice and tumors.

Female DBA/2, B6D2F<sub>1</sub>, B10.D2, B10 and C57BL/6 mice (6 to 10 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, Maine). A.TH animals were obtained from the Banting and Best Institute from Dr. T. Delovitch and were bred in our own animal unit. P815 mastocytoma and L1210 leukemia were obtained from Dr. Bruce Smith (Institute for Cancer Research, Philadelphia, Pennsylvania) and maintained as an ascites tumor as described previously (11,12).

### Culture system for generation of cytotoxic effector cells.

Single-cell suspensions were prepared from spleens of normal DBA/2 mice by pressing the tissue through a stainless steel 60-gauge mesh in RPMI 1640 (Grand Island Biological Company, Grand Island, N.Y.) medium containing 10% heat-inactivated fetal calf serum (FCS), 10 mM HEPES and  $5 \times 10^{-5}$  mM of 2-mercaptoethanol. Gentamycin was also added to a final concentration of 50 g/ml. Suspended cells were washed through 3.0 ml of FCS, resuspended in medium, and counted for viable cells by using trypan blue. Tumor cells were washed twice in medium before resuspension in complete medium for counting. Specific cytotoxicity against either P815 or L1210 was generated in vitro by incubating  $10^7$  splenic lymphoid cells with  $5 \times 10^5$  mitomycin C-treated tumor cells in Linbro multiwell plates containing 24 flat bottom wells (No. 76-033-05) at 37°C in a humidified incubator with 5% CO<sub>2</sub> for 5 days. The total volume of each culture was 2.5 ml. After

5 days, cells were harvested by centrifugation, washed, and viable cells were counted by trypan blue exclusion and tested for cytotoxicity.

Assay for suppressor cells and suppressor factors.

When the assay was used for measuring suppressor cell activity,  $5 \times 10^6$  normal splenic lymphocytes were incubated with  $5 \times 10^6$  suppressor thymocytes; a control contained  $5 \times 10^6$  normal splenic lymphocytes and  $5 \times 10^6$  normal thymocytes. On all occasions, experiments were run at least in duplicate with lymphocytes from individual pools of cells in order to minimize errors incurred by inaccurate counts. Assay for thymic suppressor factor involved incubation of  $10^7$  splenic lymphocytes with various concentrations of the factor under the same conditions as stated above.

When the degree of suppression was quantitated, cytotoxicity was tested at various effector to target cell ratios and the decrease in total lytic units was assessed. One lytic unit, which was defined as the number of effector cells required to lyse 50% of the  $10^4$  target cells in 18 hr incubation, was estimated by linear regression analysis of percentage of cytotoxicity vs logarithm of effector to target cell ratio. Total lytic units were calculated from cell recovery in cultures and percentage of suppression was estimated from the decrease in total lytic units. No significant differences in cell recovery between test cultures and controls were normally observed.

When thymocytes on their own, at a concentration of  $10^7$  per well were cultured with micomycin-treated P815, no detectable killer cells were generated, indicating that in the syngeneic killing system, thymocytes do not contain significant levels of precursors. This was also found to be the case when  $5 \times 10^6$  thymocytes were cultured with  $5 \times 10^6$  mitomycin-treated DBA/2 spleen cells or C57BL/6 chimeric cells.

#### Cytotoxicity assay.

Target tumor cells were labeled with  $^{51}\text{Cr}$ -sodium chromate (New England Nuclear, Boston, Mass.) as described previously (11). Various numbers of lymphocytes and  $10^4$   $^{51}\text{Cr}$ -labeled target cells in RPMI 1640 culture medium were dispensed in the wells of multi-dish microculture plates (Linbro Chemical, 1S-FB-96-TC, New Haven, Conn.) and the final volume was adjusted to 0.20 ml. The plates were incubated for 18 hr in a  $\text{CO}_2$  enriched, humidified incubator at  $37^\circ\text{C}$ . The cells were then sedimented by centrifuging the plates at  $200 \times G$  for 5 min, 0.10 ml of the supernatant was removed, and its radioactivity was measured on a gamma counter (Beckman Biogamma). Percentage of specific cytotoxicity was calculated as follows:

Specific cytotoxicity (%)

$$= \frac{\text{test release (CPM)} - \text{spontaneous release (CPM)}}{\text{maximum release (CPM)} - \text{spontaneous release (CPM)}} \times 100$$

Spontaneous release was measured by incubating  $10^4$  target cells alone, and maximum release was tested by lysing  $10^4$  target cells

with 5% TRITON X 100 (Sigma Chemical Company, St. Louis, Mo.).

Spontaneous release of P815 cells was 20 to 25% and that of L1210 cells was 15 to 20% of maximum release.

#### Generation of suppressor cells.

DBA/2 mice were injected subcutaneously with  $2 \times 10^3$  P815 cells in the right flank. They were sacrificed 8 days later and their thymocytes used as a source of suppressor cells. This protocol had been shown previously (65) to be reliable and reproducible for the generation of T lymphocytes specifically suppressive in the assay system.

#### Preparation of suppressor factor.

This method has been described in detail previously (65). Briefly, suppressive thymocytes prepared as stated above, were disrupted by sonication (Biosonic 80) at  $0^\circ\text{C}$  for three 1-min bursts and the soluble material was subjected to preparative isoelectric focusing after ultracentrifugation at  $60,000 \times G$  for 60 min. By titration of individual fractions, from both normal and tumor bearer extracts, the specifically suppressive fraction was isolated. This material was stable at  $4^\circ\text{C}$  and active up to dilutions equivalent to 1/125 of a single thymus. Assays for this material were always run against controls containing equal concentrations of the equivalent fractions from normal thymocytes.

#### Isoelectric focusing.

Preparative isoelectric focusing was run on a 110 ml LKB-focusing column through a 5 to 50% linear sucrose gradient and a

total of 1.6% LKB ampholines (1.2%, pH 4 to 6, and 0.4%, pH 5 to 7). Crude thymic extracts in volumes of 2.5 to 3.0 ml with 280 nm absorbance of about 10.0/ml were used. The electrode solutions consisted of 7.0 ml  $H_2O$  and 3.0 ml 1 M NaOH (light electrode) and 15 g sucrose, 13 ml  $H_2O$  and 3 ml 1 M  $H_3PO_4$  (dense electrode). Focusing was carried out at  $4^{\circ}C$  for 24 hr at 950 volts and 0.8 mA. After the focusing was complete, distilled water was pumped slowly into the column and 1.0 ml fractions of the focused materials were collected. These fractions were monitored for pH and absorbance at 280 nm. Preliminary testing had indicated that the suppressive material focused at between pH 4.6 and 4.7. Therefore, when fractions were being pooled for subsequent testing, both pH and absorbance at 280 nm were used as indices for cutting the fractions. Pooled samples were dialyzed against physiologic saline to eliminate the sucrose and ampholines. They were then concentrated down to their original volume with an Amicon ultrafilter with a UM10 membrane. Fractions were then dialyzed against RPMI 1640 after which their absorbance at 280 nm was recorded. They were then sterilized by Millipore filtration and stored at  $4^{\circ}C$  until they were titrated or used for further characterization studies.

#### Preparation of alloantisera.

The anti-Ia<sup>d</sup> antiserum used here was prepared by immunizing B10 mice repeatedly with B10.D2 splenocytes. Animals were injected i.p. every week with  $10^7$  B10.D2 splenocytes for 8 weeks. Subsequently, they were bled from the retroorbital sinus on



alternate weeks and continually immunized over a period of 2 months. The anti-H-2<sup>d</sup> antiserum thus prepared was 100% cytotoxic to P815 cells in the presence of rabbit complement (C) up to a final dilution of 1/1200. In order to render the antiserum specific for the Ia region of the H-2<sup>d</sup> MHC it was absorbed exhaustively against P815 cells until it was no longer cytotoxic in the presence of rabbit C for these targets. The absorbed antiserum (anti-Ia<sup>d</sup>), when tested for direct killing of DBA/2 splenic lymphocytes killed 60 to 65% of these cells up to a final dilution of 1/80 but had no appreciable cytotoxicity for thymocytes at a dilution of 1/4. When tested by <sup>51</sup>Cr release assay with LPS blasts, it caused 75% <sup>51</sup>Cr release up to a 1/100 dilution. In tests reported here the anti-Ia<sup>d</sup> antisera was used at a final concentration of 1/40 in the presence of a 1/15 dilution of rabbit C (Lowtox Cedar Lane Labs, Hornby, Ontario). Antisera specific for H-2<sup>t2</sup> (A.TH anti A.TL) was obtained from Dr. T. Delovitch (Banting and Best Institute). Antiserum to H-2<sup>b</sup> haplotype was prepared by immunizing B10.D2 mice with B10 splenocytes as described above.

#### Radiation chimeras.

B6D2F<sub>1</sub> mice were lethally irradiated with 900 R and reconstituted with 10<sup>7</sup> bone marrow cells from either DBA/2, C57BL/6 or A.TH donors. The donor cells had been treated with rabbit anti-purified brain associated thy-1 antigen. The antiserum was obtained from M. Letarte (Ontario Cancer Institute) and

was used at a dilution of 1/200 with a 1/15 dilution of rabbit C. Animals were kept for 8 weeks before they were used in any experiments. When these animals were sacrificed, their spleen cells were tested with the appropriate alloantisera plus C to ascertain that their cells were those characteristic of the donor allotype.

#### Anti-Ia<sup>d</sup> killing of suppressor cells.

In experiments testing for the presence of Ia markers on suppressor cells,  $4 \times 10^7$  tumor bearer or normal thymocytes were suspended in 1.0 ml of a 1/40 dilution of antiserum in complete medium and incubated for 45 min at 37°C after which 0.5 ml of a 1/5 dilution of rabbit C in the same medium was added and incubation was continued for another 45 min. Appropriate controls of C and medium only were run concurrently. Cells were washed, resuspended in medium, and counted for viability. In no instance were viable counts in cell suspensions containing the anti-Ia<sup>d</sup> antiserum significantly different from those of the controls and in no instance were counts lower than 90% of the number of cells before treatment. These cells were subsequently used in suppressor cell assays.

#### Immunoabsorbent assays.

The ability of anti-Ia<sup>d</sup> to remove the suppressive factor from thymic extracts was tested by using an immunoabsorbent assay described previously (65). Briefly, both normal and suppressor isoelectric focused fractions were mixed with either the anti-

Ia<sup>d</sup> antiserum or a control of anti-H-2<sup>b</sup> antiserum, and incubated overnight at 4°C. These materials were then passed over immuno-adsorbent Sepharose 4B columns to which goat anti-mouse Ig had been attached by cyanogen bromide. The capacity of the column was sufficient to remove at least twice the amount of the mouse Ig added to the fractions. The material eluting from the columns was titrated for suppressive activity over dilutions ranging from 1/5 to 1/125 (these dilutions of eluted materials represent between 0.2 and 0.04 of a thymus equivalent).

## Results

The ability of suppressor cells generated as previously described (12,65) to suppress specifically the primary in vitro generation of DBA/2 cells cytotoxic for the syngeneic P815 mastocytoma is shown in Figure 1. A primary cytotoxic response was generated against either P815 or L1210 mitomycin-treated cells in the presence of either normal or suppressor thymocytes. As can be seen in Figure 1a, the response to P815 in the presence of suppressor thymocytes was significantly suppressed whereas the response to L1210 was not. It was thus shown that these suppressor cells that had been found previously (12) to suppress the generation of P815 cytotoxic cells in populations of splenocytes primed in vivo by exposure to P815, could also suppress the primary response in an analogous manner. These results are entirely reproducible, the only difference being in the relative numbers of lytic units between cultures. The data shown in Figure 1 are representative in that control cultures contain roughly twice the number of lytic units as do cultures containing suppressor cells.

It has been reported by others that specific T suppressor cells bear detectable Ia antigens on their surface (81,90-94). Experiments were run to determine whether anti-Ia<sup>d</sup> alloantiserum plus rabbit C could eliminate suppressor cells in this system. The results of a representative experiment are shown in Figure 2. Although the C control showed that suppressor cells were present in the treated population of thymocytes (Fig. 2b), the anti-Ia treatment removed suppressive activity so that the cytotoxicity generated in the test population was not significantly different from the control population contain-

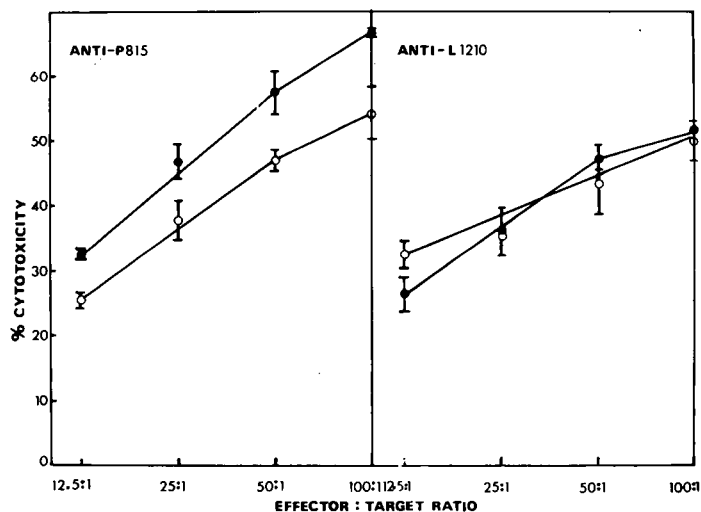


Figure 1. The ability of P815 suppressor cells to suppress the in vitro primary cytotoxic response of normal DBA/2 splenocytes to syngeneic mitomycin C-treated P815 tumor cells.

Anti-P815: the cytotoxic response of cells primed in vitro with P815 cells on  $^{51}\text{Cr}$  labeled P815 cells in an 18 hr-assay.

●—●, primary culture of normal DBA/2 splenocytes and normal DBA/2 thymocytes (lytic units were 33.3); ○—○, primary culture of normal DBA/2 splenocytes and P815 suppressor thymocytes (lytic units were 15.4).

Anti-L1210: the cytotoxic response of cells primed in vitro with L1210 cells on  $^{51}\text{Cr}$  labeled L1210 cells in an 18 hr assay. ●—●, primary culture of normal DBA/2 splenocytes and normal DBA/2 thymocytes; ○—○, primary culture of normal DBA/2 splenocytes and P815 suppressor thymocytes. Lytic units for both cultures were 11.1.

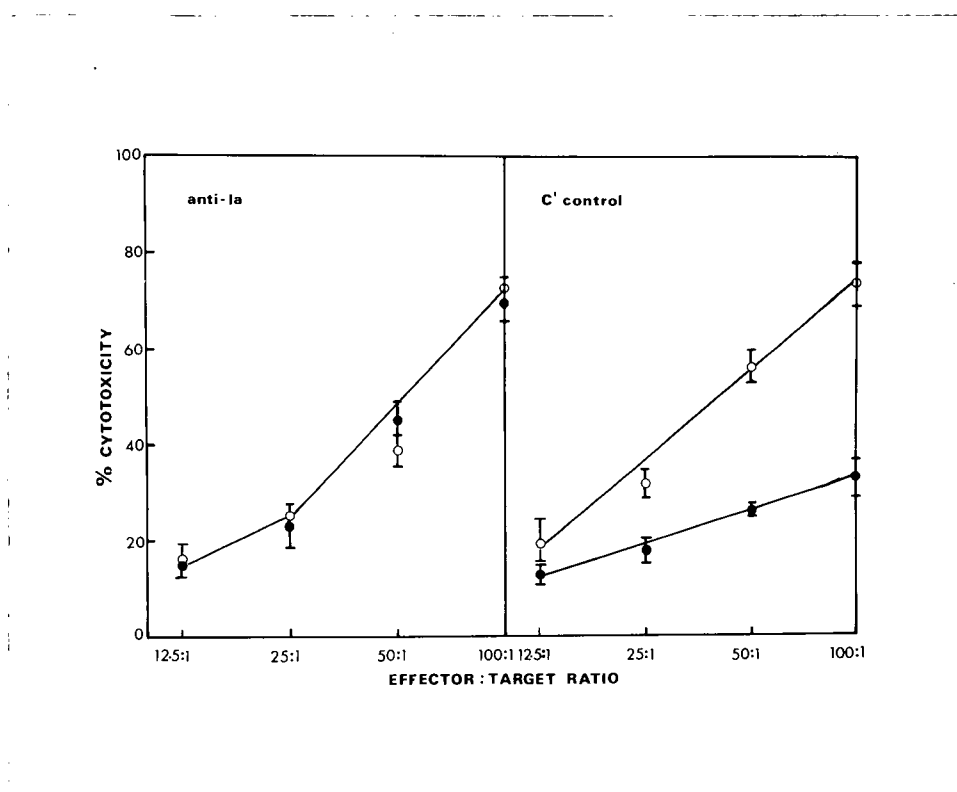


Figure 2. The ability of anti-Ia<sup>d</sup> alloantiserum plus rabbit C to eliminate P815 suppressor cells.

Anti-Ia: the cytotoxic response of cells primed *in vitro* with P815 after treatment of thymocytes with anti-Ia<sup>d</sup> anti-serum plus C. ○—○, primary culture of normal DBA/2 splenocytes plus anti-Ia- treated normal thymocytes; ●—●, primary culture of normal DBA/2 splenocytes plus anti-Ia<sup>d</sup> treated suppressor thymocytes. Lytic units in both cultures were 19.2.

C control: C control of the anti-Ia<sup>d</sup> experiment ○—○, primary culture of normal DBA/2 splenocytes and C-treated normal thymocytes (lytic units were 24.4); ●—●, primary culture of normal DBA/2 splenocytes and C-treated P815 suppressor thymocytes. Lytic units for suppressor cell populations in this instance could not be calculated because 50% lysis of cells was not achieved in the assay.

ing anti-Ia-treated normal thymocytes (Fig. 2a).

The potential of suppressor cells to operate across a histocompatibility barrier was tested by using spleen cells from B6D2 radiation chimeras that had been reconstituted with DBA/2, C57BL/6, or A.TH bone marrow cells, thus providing cells of various H-2 haplotypes rendered tolerant to H-2<sup>d</sup> alloantigens. These were then cultured with suppressor cells generated in vivo in DBA/2 mice plus mitomycin C-treated P815 cells. The results are shown in Figures 3 and 4. In Figure 3, the suppressive activity of these cells when cocultured with spleen cells of either C57BL/6 or DBA/2 allotype was essentially the same, indicating that suppressor cells do not require K or D identity in order to carry out their suppressive effect. These experiments have been repeated a number of times and are totally reproducible. Similar experiments were run with chimeras reconstituted with A.TH cells (Fig. 4). This haplotype shares the D locus with DBA/2 but differs at all other regions of the MHC (H-2<sup>t2</sup>). As can be seen, again there was no difference in the suppressive activity of the DBA/2 suppressor cells with either H-2<sup>t2</sup> or H-2<sup>d</sup> splenocytes. All the spleen cells from the chimeras were tested with appropriate antisera to ensure that they were the haplotype of the donor cells.

The comparative properties of the suppressor cells and the suppressive factor separated from the suppressor cell populations were studied. Figure 5 shows the ability of suppressor factor to suppress specifically the primary in vitro generation of DBA/2 cells cytotoxic for the syngeneic P815 mastocytoma. As can be seen in Figure 5a, the response to P815 in the presence of suppressor factor

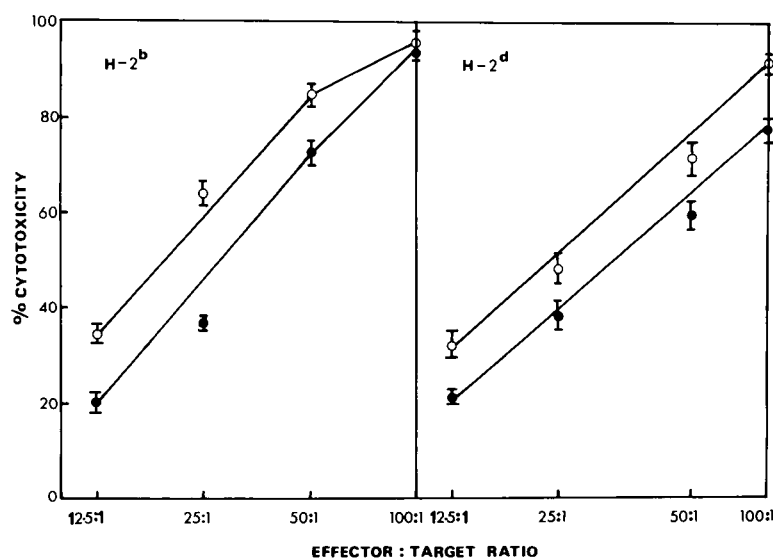


Figure 3. The ability of DBA/2 P815 suppressor cells to suppress the cytotoxic response of splenocytes of H-2<sup>b</sup> radiation chimeras to P815.

H-2<sup>b</sup>: the cytotoxic response of H-2<sup>b</sup> (C57BL/6 reconstituted) radiation chimera splenocytes, primed in vitro with P815. ○—○, primary culture of H-2<sup>b</sup> chimera splenocytes and normal DBA/2 thymocytes (lytic units were 69.0); ●—●, primary culture of H-2<sup>b</sup> chimera splenocytes and P815 suppressor thymocytes (lytic units were 35.7).

H-2<sup>d</sup>: control assay for suppressor cells. ○—○, primary culture of H-2<sup>d</sup> (DBA/2) chimera splenocytes and normal DBA/2 thymocytes (lytic units were 41.7); ●—●, primary culture of H-2<sup>d</sup> chimera splenocytes and P815 suppressor thymocytes (lytic units were 27.4).



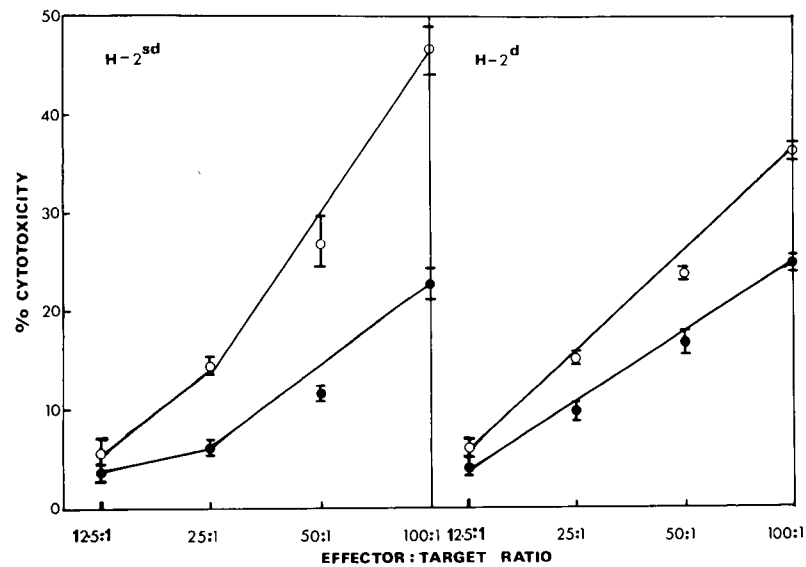


Figure 4. The ability of DBA/2 P815 suppressor cells to suppress the cytotoxic response of splenocytes from H-2<sup>t2</sup> (A.TH) radiation chimeras to P815.

H-2<sup>t2</sup>: the cytotoxic response of H-2<sup>t2</sup> radiation chimera splenocytes primed in vitro with P815. O—O, primary culture of H-2<sup>t2</sup> chimera splenocytes and normal DBA/2 thymocytes (lytic units were 10.9); ●—●, primary culture of H-2<sup>t2</sup> chimera splenocytes and P815 suppressor thymocytes (lytic units could not be calculated because of low levels of cytotoxicity).

H-2<sup>d</sup>: control assay for suppressor cells. O—O, primary culture of H-2<sup>d</sup> chimera splenocytes and normal DBA/2 thymocytes; ●—●, primary culture of H-2<sup>d</sup> chimera splenocytes and P815 suppressor thymocytes (Lytic units could not be measured in this experiment).

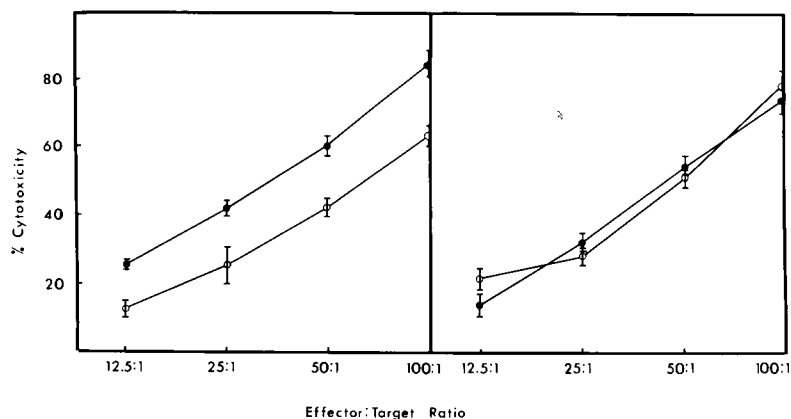


Figure 5. The ability of the suppressor factor isolated from P815 suppressor thymocyte populations to suppress the in vitro primary cytotoxic response of normal DBA/2 splenocytes to syngeneic mitomycin C-treated tumor cells. The dilutions used in this assay represent approximately 1/25 of a single thymus.

Anti-P815: the cytotoxic response of cells primed in vitro with P815 cells on  $^{51}\text{Cr}$  labeled P815 cells in an 18 hr-assay. ●—●, primary culture of normal DBA/2 splenocytes and normal DBA/2 thymocyte extract (lytic units were 59.1); ○—○, primary culture of normal DBA/2 splenocytes and P815 suppressor thymocyte extract (lytic units were 31.8).

Anti-L1210: the cytotoxic response of cells primed in vitro with L1210 cells on  $^{51}\text{Cr}$  labeled L1210 cells in an 18 hr-assay. ●—●, primary culture of normal DBA/2 splenocytes and normal DBA/2 thymocyte extract (lytic units were 45.6) ○—○, primary culture of normal DBA/2 splenocytes and P815 suppressor thymocyte extract (lytic units were 44.8).

was significantly suppressed whereas the response the Ll2l0 was not.

Experiments were run to determine whether adsorption with anti-Ia<sup>d</sup> alloantiserum could eliminate suppressor factor activity in our system. Partially purified factor and the equivalent fraction of normal thymocyte extracts were subjected to treatment with anti-Ia<sup>d</sup> antiserum or anti-H-2<sup>b</sup> antiserum and adsorbed on immunoadsorbent columns containing anti-mouse immunoglobulin. The eluted materials were titrated for suppressive activity. The results are shown in Figure 6. While suppressive activity was present in the anti-H-2<sup>b</sup> suppressor factor eluates, the suppressive material treated with anti-Ia<sup>d</sup> was not, showing that it expresses determinants encoded by the Ia region of the MHC.

The ability of the factor, when added to B6D2F<sub>1</sub> C57BL/6 chimeric splenocytes, to suppress the generation of cells cytotoxic to P815, was also tested. The results (Fig. 7) show, as with the suppressor cells, that the suppressive activity of the factor with H-2<sup>b</sup> cells was similar to that observed with the DBA/2 cells tested at the same time.

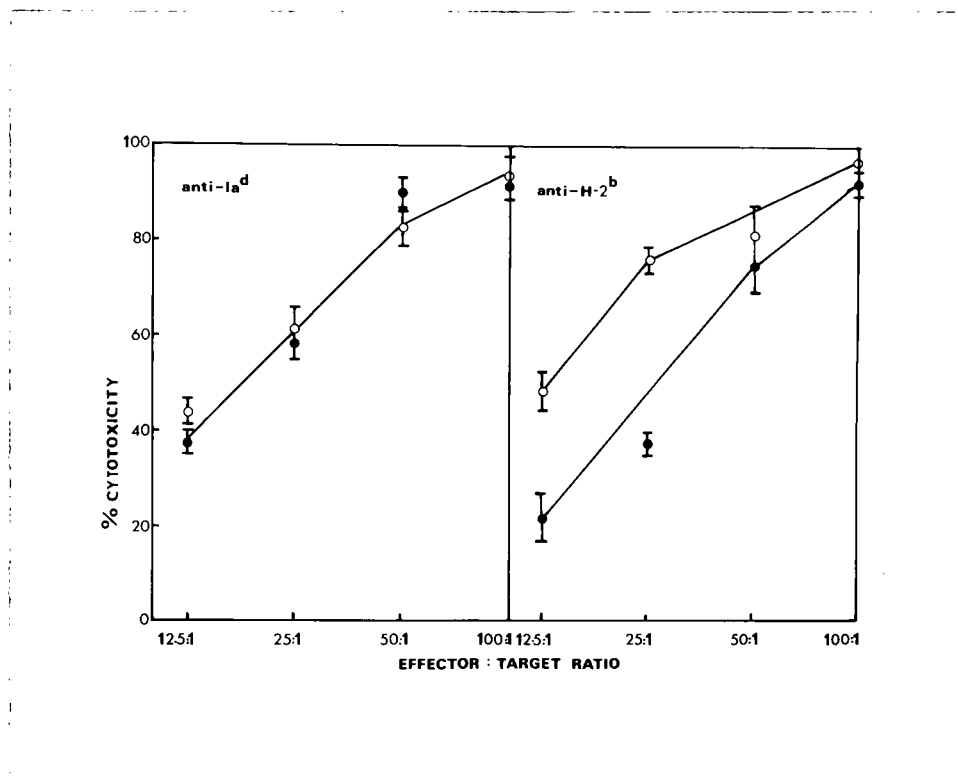


Figure 6. The effect of anti-Ia antiserum adsorption on the suppressive activity of the suppressor factor isolated from P815 suppressor thymocyte populations. The dilutions used in this assay represent approximately 1/25 of a single thymus.

Anti-Ia<sup>d</sup>: the effect of adsorption of the suppressive factor with anti-Ia<sup>d</sup> antiserum. ○—○, primary culture of normal DBA/2 splenocytes and anti-Ia<sup>d</sup> adsorbed normal thymocyte extracts; ●—●, primary culture of normal DBA/2 splenocytes and anti-Ia<sup>d</sup> adsorbed suppressor thymocyte extracts. Lytic units in both cases were 57.1.

Anti-H-2<sup>b</sup>: the effect of adsorption of the suppressive factor with anti-Ia<sup>b</sup> antiserum. ○—○, primary culture of normal DBA/2 splenocytes and anti-Ia<sup>b</sup> adsorbed normal thymocyte extract (lytic units were 76.9); ●—●, primary culture of normal DBA/2 splenocytes and anti-Ia<sup>b</sup> adsorbed suppressor thymocyte extract (lytic units were 38.5).

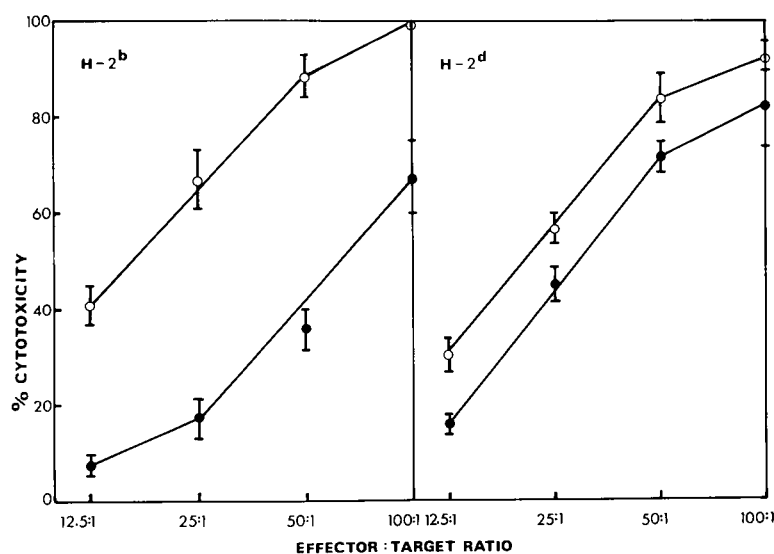


Figure 7. The ability of P815 suppressor factor to suppress the cytotoxic response of splenocytes from H-2<sup>b</sup> radiation chimeras to P815. The amount of factor used in these experiments was approximately 1/25 thymus equivalent.

H-2<sup>b</sup>: the cytotoxic response of H-2<sup>b</sup> radiation chimera splenocytes primed *in vitro* with P815. ○—○, primary culture of H-2<sup>b</sup> chimera splenocytes and normal thymocyte extract (lytic units were 62.5); ●—●, primary culture of H-2<sup>b</sup> chimera splenocytes and suppressor factor (lytic units were 16.1).

H-2<sup>d</sup>: control assay for suppressor factor. ○—○, primary culture of H-2<sup>d</sup> chimera splenocytes and normal thymocyte extract (lytic units were 48.8); ●—●, primary culture of H-2<sup>d</sup> chimera splenocytes and suppressor factor (lytic units were 33.9).

## Discussion

In previous studies on suppressor cells and the specific factors possibly released from them, it was demonstrated that such suppressive elements were capable of specifically suppressing the generation of cytotoxic cells in vitro by spleen cells from P815-primed animals (11,12,65). In this chapter, it was shown that P815-specific suppressor cells and P815 specific suppressor factor are also capable of suppressing a primary in vitro cytotoxic response to P815 (Fig. 1,5). Thus, the state (primed or virgin) of the cell population on which it acts appears to be unimportant for the P815-specific suppressor cell or P815-specific suppressor factor to express their activity.

The ability of alloantisera directed to the Ia region (the I-J subregion in most studies) to kill suppressor T cells has been reported by others (81,90-93). These studies involved mainly the characterization of T suppressor cells participating in the regulation of the antibody response to soluble antigens. The suppressor cells studied here are those involved in regulating the generation of cytotoxic cells. However, they also appear to possess surface markers recognized by anti-haplotype sera to the Ia region, so in this respect they do not differ from other populations of antigen-specific T suppressor cells. Since at this time it is not possible to develop an antiserum specific for the I-J subregion of the Ia<sup>d</sup> haplotype, it was not possible to test the cells for this specificity although many other studies have shown that this subregion of the Ia is expressed on antigen-specific suppressor T cells.

In this chapter it was undertaken to determine whether the population of T suppressor cells under study was H-2 restricted. Because the immune response being assessed for suppression involved the development of cells cytotoxic for a syngeneic tumor cell line, it was necessary to use spleen cells from radiation chimeras that were tolerant to normal H-2<sup>d</sup> alloantigens. Thus B6D2F<sub>1</sub> animals (H-2<sup>b</sup> x H-2<sup>d</sup> F<sub>1</sub>) were irradiated and reconstituted with either parental bone marrow cells (C57BL/6 or DBA/2) or with A.TH cells (H-2<sup>t2</sup>) that shares only the D locus with H-2<sup>d</sup>. Suppressor cells were raised in DBA/2 tumor bearers and then cultured in vitro with mitomycin C-treated P815 cells and chimeric spleen cells of either DBA/2, C57BL/6, or A.TH origin. The experiments, which were repeated three times showed unequivocally that the suppressor cells were capable of suppressing the anti-P815 cytotoxic response of spleen cells differing at the K (A.TH) or both the K and D locus (C57BL/6) and thus did not exhibit the classical form of H-2 restriction. This does not exclude the possibility that some form of Ia recognition was required since these chimeric cells had been influenced by the thymus of the irradiated B6D2 recipients, but does show that K or D identity is not essential for this type of interaction.

The suppressor factor(s) isolated from the suppressor cell thymocyte population and partially purified by preparative isoelectric focusing appeared to share the same properties as the suppressor cells in that it could specifically suppress the primary in vitro cytotoxic response of DBA/2 spleen cells (Fig. 5) and could be specifically removed by anti-Ia<sup>d</sup> alloantiserum (Fig. 6). In this

respect this factor is not different from factors described by others (77,81,82,86,89). As mentioned above, it was not possible to test the subregion specificity with the H-2<sup>d</sup> system.

The data reported so far on the H-2 restrictions observed in suppressive factors are somewhat equivocal. In a series of reports by Tada and his associates (78,95,96) involving suppressive factor(s) that control the antibody response, it was found that this factor required a syngeneic target cell (presumably a helper T cell) before it could effectively suppress the antibody response. Moorhead, who produced a specific suppressor factor in vitro that could suppress the development of delayed-type hypersensitivity to DNFB in mice, found that this material was H-2 restricted and that identity at either the K or D end of the MHC was sufficient to permit suppression in recipient mice (79,80,97). A I<sub>E</sub> class specific suppressor factor also shows syngeneic MHC restriction of action (66). Alternately, Waltenbaugh and his colleagues (98) in studying a soluble factor that suppresses the antibody response to the synthetic copolymer L-glutamyl<sup>50</sup>-L-tyrosine<sup>50</sup> (GT) showed that a suppressive factor, elicited in mice by injection of GT alone, could effectively suppress the response to GT coupled to carrier protein across allogeneic barriers. Similar MHC unrestricted activity is found in the GAT system (99). A SRBC specific suppressor factor of a T-hybrid line has also recently been shown to act across allogeneic barriers (100). Kontiainen and colleagues also observed no H-2 restriction of their suppressor factors (101).

The results here show that the suppressor cells under study (and the presumed equivalent factor), are capable of suppressing the



generation of cytotoxicity to P815 by histoincompatible cells. The results show clearly that the K or D gene products do not have to be shared by the suppressor cell (or its factor) and its target. Because radiation chimeras, in which the recipients were B6D2F<sub>1</sub> animals, were used for testing restriction, the conclusions can only extend to a lack of restriction at the K and D loci of the MHC since thymic influence by the recipient may have influenced recognition mechanisms on the part of the chimeric cells.

### CHAPTER III

THE LYT PHENOTYPE OF CELLS INVOLVED IN THE  
CYTOTOXIC RESPONSE TO SYNGENEIC TUMOR AND OF  
TUMOR-SPECIFIC SUPPRESSOR CELLS AND IMPROVED  
PROCEDURES IN THE GENERATION OF TUMOR-SPECIFIC  
SUPPRESSOR CELLS

## CHAPTER III

Introduction

Antigenic determinants found on lymphocyte cell surface molecules can be used as markers for studying the relationship of the expression of these determinants and cellular function. Thus, in the last few years, many studies have examined cell surface antigenic determinants which could be used to study and characterize distinct subsets of T cells. One of the most widely studied antigenic systems of T cells has been the one determined by the Ly-1 and Ly-2 loci in the mouse (102). Since these antigens appear to be expressed selectively on various subsets of undifferentiated and differentiated T cells it has been suggested that they be called Lyt antigen (103). The gene controlling the expression of Lyt-1 antigen is found on chromosome 19, whereas the gene controlling Lyt-2 expression is found on chromosome 6 (104). Two allelic forms,  $\text{Lyt}^a$  and  $\text{Lyt}^b$ , of the Lyt-1 and Lyt-2 antigens have been found and are designated Lyt-1.1, -2.1 and Lyt-1.2, -2.2 respectively (104).

A major part of the Lyt work has been carried out in mice bearing the  $\text{Lyt}^b$  allele. In such animals a number of basic observations have been made. Killer cells raised against allogeneic cells (allogeneic killer cells) have been designated as  $\text{Lyt-1}^{-}\text{2}^{+}$  (105-107). Recently, it has been reported that allogeneic effector cells with this allele may be of the  $\text{Lyt-1}^{+}\text{2}^{+}$  phenotype (108-110). This apparent discrepancy may be accounted for possibly on a quantitative rather than qualitative basis. Killer cells raised against syngeneic tumor cells (syngeneic killer cells) have also been reported to express the  $\text{Lyt-1}^{+}\text{2}^{+}$  phenotype (107,111,112).

Helper cells have been described by a number of investigators as expressing the  $\text{Lyt-1}^+2^-$  phenotype (114-116). Antigen-specific suppressor cells isolated from antigen-primed mice have been identified as  $\text{Lyt-1}^-2^+$  (115). Suppressor cells generated in vitro by concanavalin A were also found to be  $\text{Lyt-1}^-2^+$ , while the helper cells thus generated were  $\text{Lyt-1}^+2^-$  (114,117).

There have been fewer studies carried out on mice bearing the  $\text{Lyt}^a$  allele. In these studies allogeneic killer cells have been identified as  $\text{Lyt-1}^+2^+$  (108,118). Killers of syngeneic tumor cells in C3H mice were found to be predominantly  $\text{Lyt-1}^-2^+$  although it was indicated that a population of  $\text{Lyt-1}^+2^+$  cells might also be involved (119). In the case of suppressor cells, it was found that two distinct populations of cells were present, those which suppressed the delayed type hypersensitivity reaction and were  $\text{Lyt-1}^+2^-$  and those which suppressed a humoral response and were  $\text{Lyt-1}^-2^+$  (120).

It has also been reported that in vitro-induced suppressor cells in CBA mice are  $\text{Lyt-1}^-2^+$  (118), while the helper cells are  $\text{Lyt-1}^+2^+$  (118,121).

The present study was carried out in DBA/2 mice ( $\text{Lyt-1.1, -2.1}$ ) and involved the response of their cells to the syngeneic mastocytoma P815. The  $\text{Lyt}$  phenotypes of in vitro-generated allogeneic killers, syngeneic killer cells, and in vivo-generated T suppressor cells were characterized using anti- $\text{Lyt-1.1}$  and anti- $\text{Lyt-2.1}$  serum.

## Materials and Methods

### Experimental Animals and Tumors

Female DBA/2J mice (H-2<sup>d</sup>, Lyt-1.1, -2.1) and C57BL/6 mice (H-2<sup>b</sup>, Lyt-1.2, -2.2) (The Jackson Laboratories, Bar Harbor, Maine) between the ages of 2 and 5 months of age were used exclusively. The tumor lines used were the P815 mastocytoma and L1210 leukemia, both syngeneic for DBA/2J mice, and both maintained and transplanted as ascites tumors in DBA/2J mice, or as frozen cultures maintained at -70°C in liquid nitrogen. The methods of tumor maintenance have been described elsewhere (11). EL-4 lymphoma, syngeneic for C57BL/6 mice was maintained similarly.

### Cells

Single-cell suspensions were prepared from spleens of DBA/2 mice by pressing the tissue through a stainless steel 60-gauge mesh in RPMI 1640 (Grand Island Biological Company, Grand Island, N.Y.) medium containing 10% heat-inactivated fetal calf serum (FCS), 10 mM Hepes buffer, and  $5 \times 10^{-5}$  mM of 2-mercaptoethanol. Gentamycin was also added to a final concentration of 50 µg/ml. Suspended cells were washed through 3.0 ml of FCS, resuspended in complete medium, and counted for viable cells by using trypan blue. Tumor cells were washed twice in medium before resuspension in complete medium for counting.

### Culture system for the generation of cytotoxic effector cells

#### Allogeneic effectors

The method for the in vitro generation of allogeneic killer cells have been described in detail elsewhere (122).

Briefly,  $5 \times 10^6$  spleen cells from DBA/2J mice were cultured for 4 days in the presence of  $5 \times 10^6$  mitomycin-treated spleen cells from C57BL/6 mice in 24-well Linbro trays in 2.5 ml RPMI 1640 medium supplemented with 10% FCS, 10 mM Hepes buffer,  $5 \times 10^{-5}$  M 2-mercaptoethanol, and 50 g/ml gentamycin. Cells were harvested at 4 days, counted by trypan blue exclusion, and assayed for their ability to kill  $^{51}\text{Cr}$ -labeled EL4 cells in a standard 4 hr assay, using target: effector cells from 30:1 to 3.75:1.

#### Syngeneic effectors

The method by which a primary in vitro cytotoxic response to syngeneic tumor cells can be generated has been described elsewhere (113). The method is basically that described above with the exception that  $5 \times 10^6$  DBA/2 spleen cells were cultured for 5 days with  $5 \times 10^5$  mitomycin-treated P815 cells. Harvested cells were run in the standard  $^{51}\text{Cr}$  release assay for 18 hr on labeled P815 cells with effector: target ratios of between 100:1 and 12.5:1. Previous work in this laboratory has demonstrated that the killer cells generated in this way are specific for the stimulator cells (P815 in this instance) (123,124). (Also see Table I).

#### Cytotoxicity test

This has been described in detail in Chapter II.

#### Generation of suppressor cells

In most of the previous work carried out in this laboratory, antigen-specific T suppressor cells involved in regulation of the cytotoxic response of syngeneic mice to the P815 tumor line (123,124).

Table I. Specificity of cytotoxicity generated in vitro

<u>In vitro culture</u>		<u>Cytotoxicity Assay<sup>a</sup></u>	
<u>Responding Cells</u>	<u>Stimulating cells</u>	<u>Target cells</u>	<u>% cytotoxicity</u>
Normal DBA Spleen	P815	P815	56.4 ± 2.5
Normal DBA Spleen	P815	L1210	6.8 ± 2.6
Normal DBA Spleen	P815	EL-4	5.7 ± 3.0
Normal DBA Spleen	C57BL/6	P815	0.7 ± 0.6
Normal DBA Spleen	C57BL/6	L1210	5.8 ± 3.2
Normal DBA Spleen	C57BL/6	EL-4	61.2 ± 2.9

<sup>a</sup><sup>51</sup>Cr release after 18 hr incubation, effector: target ratio of 100:1 for P815 stimulated cells. <sup>51</sup>Cr release after 4 hr incubation, effector: target ratio of 715:1 for EL-4 stimulated cells.

have been obtained from the thymuses of animals into which  $3 \times 10^3$  tumor cells had been injected subcutaneously 8-9 days previously (65). The suppressor cells used in this study were generated by the intraperitoneal injection of soluble membrane extracts of P815 cells. P815 cells, from mice bearing the tumor as ascites, were drawn from the peritoneal cavity, washed in PBS, and lysed by freezing and thawing three times in distilled water. Large membrane fragments and other cell debris were removed by centrifugation at 12,000g for 30 min. Membrane components were then pelleted by centrifugation at 105,000g for 120 min. The membrane pellet was resuspended in PBS and subjected to three 1-min bursts of ultrasound (Biosonic probe, 30 setting) at 0°C. The material was again centrifuged and the supernatant presumably containing solubilized membrane components and small membrane fragments was used. The protein content of this material was determined by the standard Lowry test. Suppressor cells in the spleens of DBA/2J mice were readily induced by intraperitoneal injection of between 80 and 200 µg protein per animal. Animals were sacrificed 4 to 5 days after antigen injection, and their spleens used as a source of suppressor cells. This method is an adaptation of that described recently for the generation of suppressor T cells to methylcholanthrene-induced sarcomas (125).

Because the spleens of these animals contained both antigen-specific and nonspecific suppressor cells (see below), it was necessary to separate these two populations. Spleen cells were passed through nylon wool columns according to the method of



Julius et al (126). The modifications used here were that cells, after application to the column, were incubated for 30 min at 37°C and then eluted at 37°C. The columns were then incubated for a further 30 min at 4°C before a second elution in cold medium. The cells from both elutions were pooled and used as a source of antigen-specific suppressor cells. This procedure utilized columns which were fairly tightly packed, containing about 12-15 ml of nylon wool through which 20 ml of supplemented medium at 37°C had been passed before use. A total of  $2 \times 10^8$  spleen cells were applied to columns of this size and approximately 20% of the cells were recovered by elution. The properties of the eluted cells were characteristic of T-cell-enriched populations in that they were 95% sensitive to anti-thy-1 serum plus complement and contained very few phagocytic cells (<1.0%). The generation of suppressor cells is illustrated in Figure 8.

#### Assay for suppressor cells

The method used to assay for suppressor cells has been described in detail elsewhere.(123). Briefly,  $5 \times 10^6$  normal spleen cells were cocultured with  $5 \times 10^6$  (or fewer in some cases) suppressor cell populations in the presence of  $5 \times 10^5$  mitomycin-treated P815 cells in 24-well Linbro trays in 2.5 ml of medium. Cells were cultured for 5 days before assay for generation of cells cytotoxic for P815. Controls in these studies constituted the use of  $5 \times 10^6$  normal spleen cells plus  $5 \times 10^6$  normal spleen or spleen-derived cells which had been treated in a manner analogous to the suppressor population.

When the degree of suppression was quantitated, cytotoxicity

Preparation of P815 Tumor-Specific  
Suppressor Cells (P815-T<sub>s</sub>)

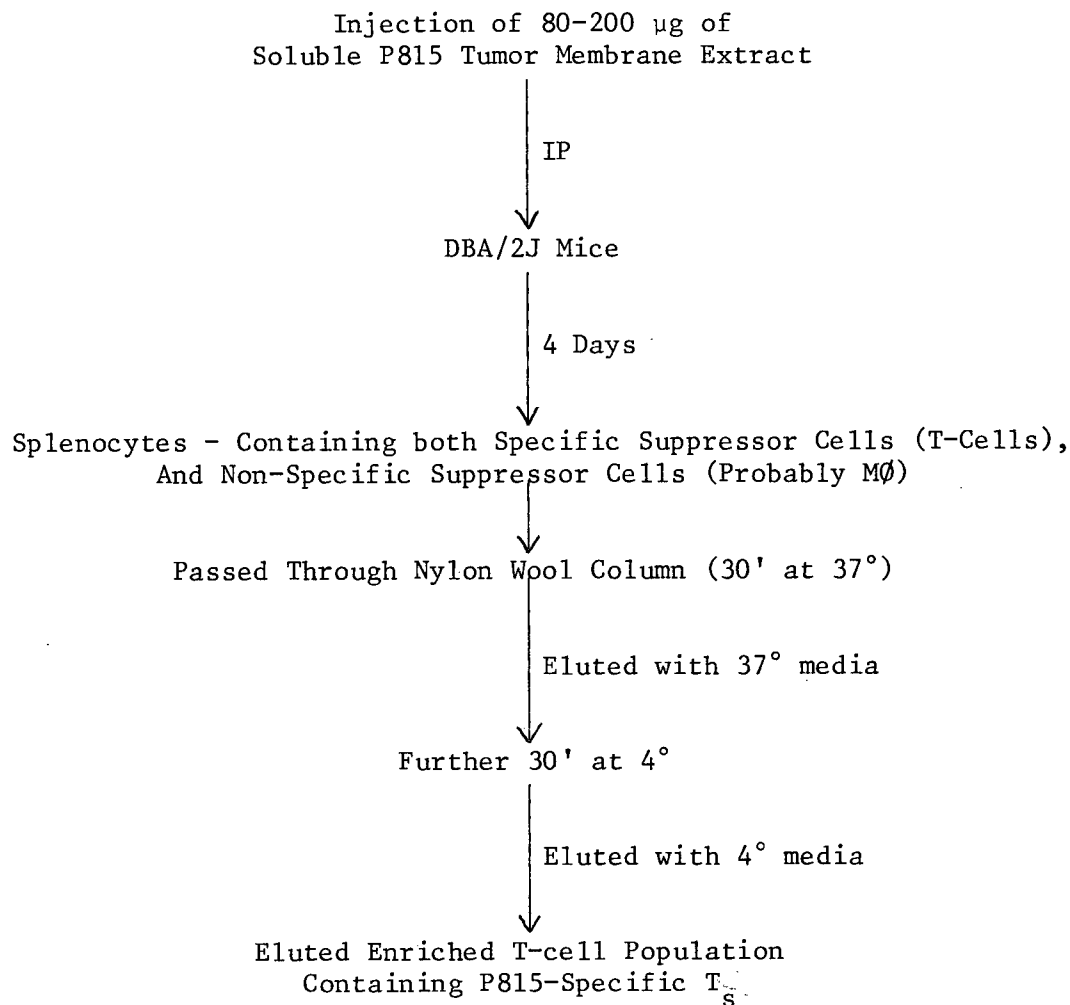


Figure 8. Flow diagram illustrating the preparation of P815 tumor-specific suppressor cells.

was tested at various effector to target ratios and the decrease in total lytic units was assessed. One lytic unit, which was defined as the number of effector cells required to lyse 50% of the  $10^4$  target cells in 18 hr incubation, was estimated by linear regression analysis of percentage of cytotoxicity vs logarithm of effector to target cell ratio. Total lytic units were calculated from cell recovery in cultures, and percentage of suppression was estimated from the decrease in total lytic units. In all experiments, no significant differences in cell recovery between test cultures and controls were observed. In all suppression experiments reported here, except the time course one (Fig. 11), a minimum of 37% suppression was seen.

#### Anti-Lyt treatment of cells

The cells tested for their Lyt phenotype in this study were: allogeneic killer cells, syngeneic killer cells, and syngeneic tumor specified suppressor cells. DBA/2J mice are of the Lyt-1.1 and Lyt-2.1 phenotype. The antisera used here were the kind gift of Dr. R. Nowinski (The Fred Hutchinson Cancer Research Center, Seattle, Washington) who had raised and characterized the antisera according the previously described procedures (127). The anti-Lyt-1.1 serum titrated at 1:640 and the anti-Lyt-2.1 titrated at 1:160 when titrated in the presence of rabbit complement on B6<sub>1</sub>Lyt-1.1 and B6Lyt-2.1, -3.1 thymocytes, respectively. The anti-Lyt-1.1 serum had no effect on B6Lyt-2.1, -3.1 thymocytes, likewise, the anti-Lyt-2.1 serum had no effect on B6.Lyt-1.1 thymocytes. In the experiments run here, higher levels of the

antisera were found to be necessary in order to kill the appropriate cells effectively. For both the allogeneic and syngeneic killer cells, the procedure was identical. Effector cells were harvested, counted, and distributed to small plastic test tubes at a total concentration of  $3.0 \times 10^7$  cells per tube. These cells were centrifuged and the supernatant removed. Three hundred microliters of medium was added to each tube and 50  $\mu$ l of either anti-Lyt-1.1, Lyt-2.1, or medium was added to the appropriate tube. The cells were mixed and incubated at room temperature for 45 min following which 50  $\mu$ l of neat rabbit complement (Low-tox, Cedar Lane, Ontario, Canada) was added to each tube except the control, to which 50  $\mu$ l of medium was added. Incubation was continued for another 45 min. Cells were washed three times in supplemented medium, counted by trypan blue exclusion, and subsequently tested for their cytotoxic activity. One population of a 1:1 mixture of anti-Lyt-1 and anti-Lyt-2-treated cells were also reconstituted and tested for cytotoxicity.

In the assay of suppressor cells, larger cell numbers had to be treated because the cells were subsequently going into the large cultures for 5 days. For this reason,  $6 \times 10^7$  cells were treated, and double the amounts of all reagents were used. A protocol as described above was used, except that after treatment, washing, and counting, the cells were put into culture at a concentration of  $5 \times 10^6$  with  $5 \times 10^6$  normal spleen cells plus mitomycin-C treated P815 cells.

## Results

The spleen cells of DBA/2J mice, 4 days following intraperitoneal injection of P815 membrane extracts, were very suppressive when they were cocultured with  $5 \times 10^6$  DBA/2 spleen cells in the presence of mitomycin-treated P815 cells. The generation of cytotoxic cells in these cultures was markedly below that seen in control cultures (Fig. 9a). When these suppressor cells were assayed in culture with normal spleen cells plus mitomycin-treated L1210 cells, the generation of cells cytotoxic for this tumor were also markedly suppressed (Fig. 9b). Since these two systems do not cross react at this level, (see Fig. 1), (12) it was concluded that intraperitoneal injection of membrane extracts of P815 cells could generate both specific and nonspecific suppressor cells. Since the most common nonspecific suppressor cell is an adherent cell, probably of the macrophage-monocyte series (18,128,129), an attempt to achieve specificity in the system by passing the spleen cells from antigen-injected animals through nylon wool columns was attempted. The cells eluted from these columns at either 37 or 4°C were tested for their suppressive activity. Since both populations were found to contain specific suppressor cells, subsequent studies involved a pool of the two populations (Fig. 10).

The time course of appearance and disappearance of the antigen-specific suppressor cells was examined. As can be seen in Figure 11, DBA/2 splenocytes show antigen-specific suppression within 2 days following intraperitoneal injection of P815 membrane extracts. This suppression peaks at 4-5 days, then falls off rapidly to control levels by 7-9 days post injection.

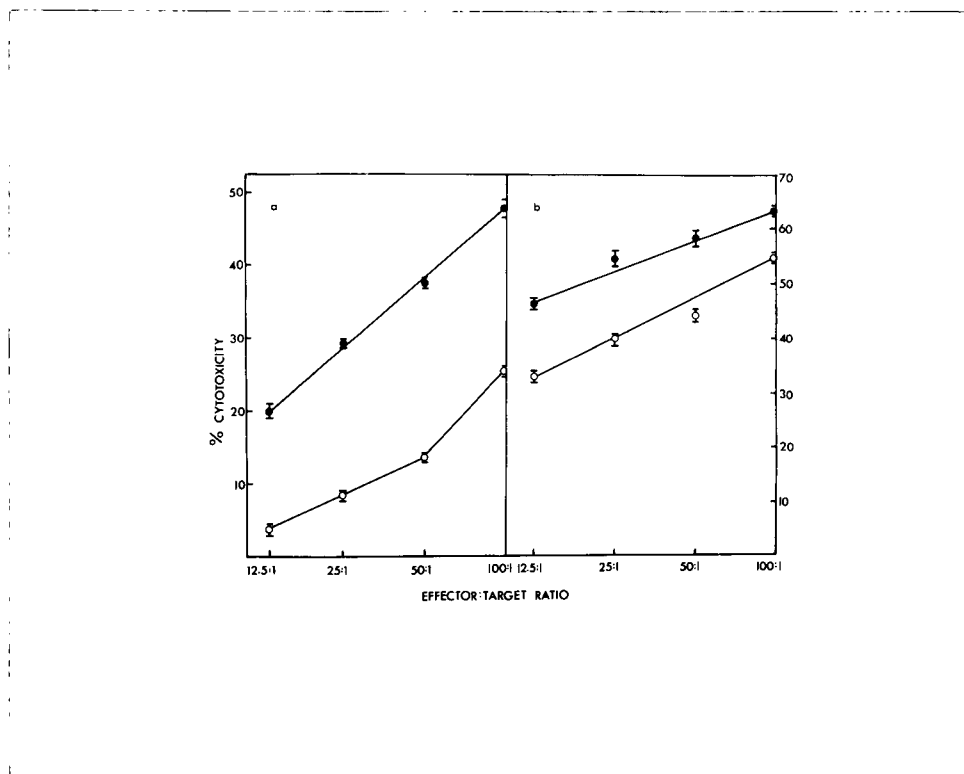


Figure 9. Cytotoxicity titrations of DBA/2 spleen cells primed against either P815 stimulator (a) or L1210 stimulators (b). (●) Control containing  $10^7$  normal DBA/2 splenocytes in the priming culture; (○) cultures containing  $5 \times 10^6$  normal DBA/2 splenocytes and  $5 \times 10^6$  splenocytes from mice inoculated 4 days previously with P815 membrane extracts (80  $\mu$ g per mouse).

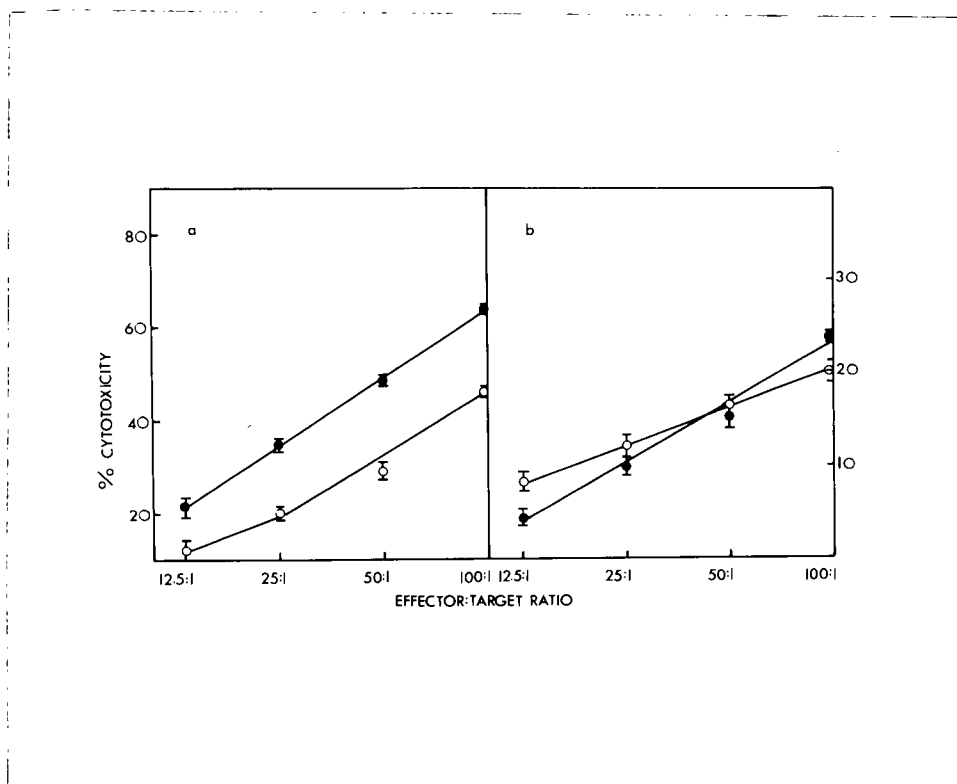


Figure 10. Cytotoxicity titrations of DBA/2 spleen cells primed against either P815 (a) or L1210 (b) stimulators. (●) Control culture containing  $5 \times 10^6$  normal DBA/2 splenocytes and  $5 \times 10^6$  normal DBA/2 nylon wool-passed splenic lymphocytes; (○) cultures containing  $5 \times 10^6$  normal DBA/2 splenocytes and  $5 \times 10^6$  P815 primed DBA/2 nylon wool-passed splenic lymphocytes (cells eluted at  $37^\circ$  and  $4^\circ\text{C}$  were pooled).

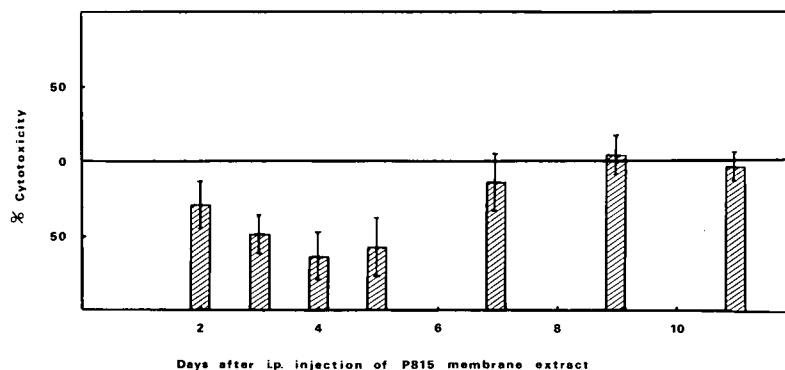


Figure 11. Time course of appearance and disappearance of antigen-specific suppressor cells in the spleen of DBA/2 mice injected intraperitoneally with P815 membrane extracts on day 0. Control cultures contained  $5 \times 10^6$  normal DBA splenocytes and  $5 \times 10^6$  normal DBA/2 nylon wool-passed splenic lymphocytes. Test cultures contained  $5 \times 10^6$  normal DBA/2 splenocytes and  $5 \times 10^6$  P815 primed DBA/2 nylon wool-passed splenic lymphocytes. Results are plotted as MEAN % suppression caused by P815 injected spleen as compared to controls.



The Lyt phenotypes of a variety of effector cells in DBA/2 mice was investigated. Since previous findings by others had characterized allogeneic killers in mice of the  $\text{Lyt}^a$  allele as  $\text{Lyt-1}^{+2+}$ , it was considered important to test the antisera by determining its effect on allogeneic killer cells. The results are shown in Figure 12, in which it can be seen that both anti-Lyt-1.1 and anti-Lyt-2.1 plus complement effectively abrogated the allogeneic killer cells. Mixing of anti-Lyt-1.1 and anti-Lyt-2.1-treated cells prior to the cytotoxicity assay did not reconstitute the response (data not shown). These results, therefore, are in agreement with the observations of others and indicate that the allogeneic killer cell in the DBA/2J animal expresses the  $\text{Lyt-1}^{+2+}$  phenotype (108,118).

An experiment was run concurrently to determine the Lyt phenotype of the cytotoxic effector to syngeneic tumor cells. The results are shown in Figure 13. In this case complement plus anti-Lyt-2.1 but not anti-Lyt-1.1 reduced the killing efficiency of this population, indicating that the Lyt phenotype of this cell is  $\text{Lyt-1}^{-2+}$ . Mixing of the two treated populations did not restore the response.

Finally, the Lyt phenotype of the DBA/2 antigen-specific suppressor cell was investigated. The results are shown in Figure 14. In this instance, the anti-Lyt-1 antiserum eliminated suppressor cells, or their development, whereas the anti-Lyt-2 serum had no effect. In this case also, mixtures of anti-Lyt-1 and Lyt-2-treated cells did not reconstitute the response. It would therefore appear, in this system, that the suppressor cell, its progenitor, or a cell vital in its development expresses the  $\text{Lyt-1}^{+2-}$  phenotype.

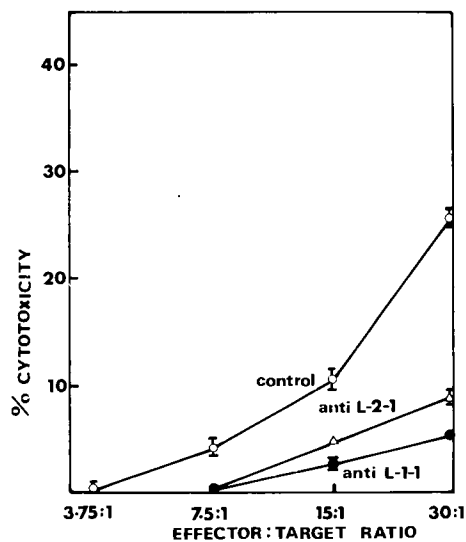


Figure 12. The effect of anti-Lyt-1.1 and anti-Lyt-2.1 serum plus rabbit C' on DBA/2 allogeneic effector cells raised against C57BL/6, on a EL4 target. (○) Control culture; (●) anti-Lyt-1.1-treated effector cells; (Δ) anti-Lyt-2.1-treated effector cells.

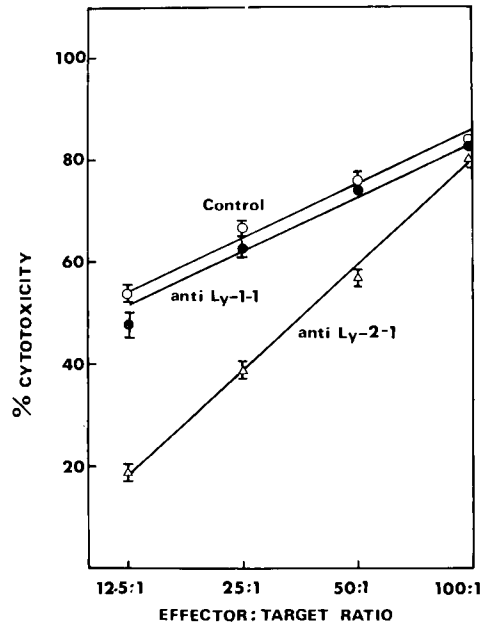


Figure 13. The effect of anti-Lyt-1.1 and anti-Lyt-2.1 serum plus rabbit C' on DBA/2 effector cells primed against the syngeneic P815 mastocytoma. (○) Control; (●) anti-Lyt-1.1-treated effector cells; (Δ) anti-Lyt-2.1-treated effector cells.

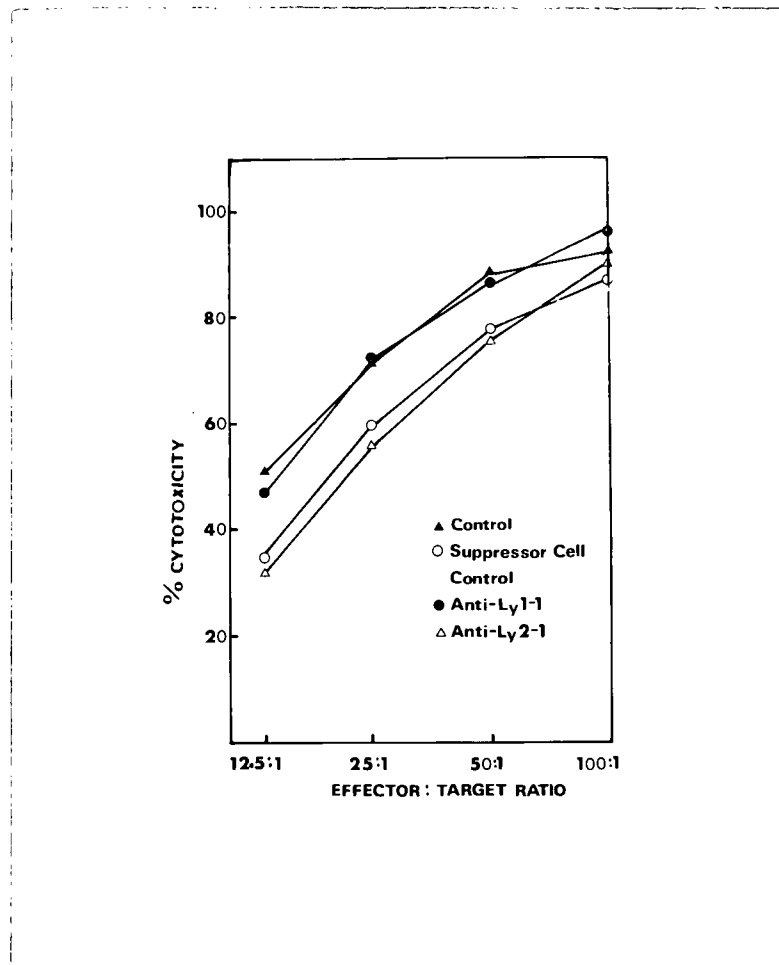


Figure 14. The effect of anti-Lyt-1.1 and anti-Lyt-2.1 serum plus rabbit C' on P815-primed nylon wool-passed DBA/2 splenic suppressor cells. (▲) Control cultures containing  $5 \times 10^6$  normal DBA/2 cells plus  $5 \times 10^6$  nylon wool-passed normal DBA/2 splenocytes; (○) positive control culture containing  $5 \times 10^6$  normal DBA/2 splenocytes plus  $5 \times 10^6$  nylon wool-passed P815 suppressor cells; (●) culture containing  $5 \times 10^6$  normal DBA/2 splenocytes plus  $5 \times 10^6$  anti-Lyt-1.1-treated P815 suppressor cells; (△) culture containing  $5 \times 10^6$  normal DBA/2 splenocytes plus  $5 \times 10^6$  anti-Lyt-2.1-treated P815 suppressor cells. The SEM in this experiment was always  $<3.0\%$  and therefore not shown. Cell recoveries of antiserum-treated suppressor T cell populations were as follows: anti-Lyt-1.1, 68.7%; anti-Lyt-2.1, 70.3%. These data are based on estimates from the complement-treated control cells which were regarded as 100% recovery.

Lytic units were: (▲) 84.3, (○) 46.1, (●) 78.6, (△) 41.7.

## Discussion

In this chapter, a method for the in vivo induction of specific suppressor cells which inhibit the in vitro generation of cells cytotoxic for a syngeneic tumor has been described. The method is similar to the method described by Fujimoto for the induction of the same type of suppressor cells with a different tumor (125). However, in the work shown here, the suppression was not specific when whole spleen cell populations of tumor antigen-injected mice were used, as indicated by their ability to suppress the in vitro cytotoxic response to L1210 leukemia cells as well as P815 mastocytoma cells. Since it is well documented that antigen-stimulated spleen cells may contain a population of nonspecific suppressor cells which exhibit the characteristics of adherence to plastic or nylon wool and phagocytic activity (18,128, 129), it was felt that the passage of the stimulated spleen cells through nylon wool might remove the nonspecific suppressive activity. This proved to be the case. That the antigen-specific suppressor cell was of thymic origin has been shown previously (12) and is implicit in the finding that the nylon wool nonadherent population (which is >95% thy-1 positive) contained the antigen-specific suppressor cell.

The studies on the Lyt phenotypes of the various effector cells in this in vitro system were informative. The finding that the allogeneic killer cell in the DBA/2J mouse was  $\text{Lyt-1}^{+}\text{2}^{+}$  was not surprising, since it confirms the observations of others that, in mice bearing the Lyt-1.1 and 2.1 allele, the allogeneic killer cell is indeed  $\text{Lyt-1}^{+}\text{2}^{+}$  (108,118). Very little information is available regarding syngenic killer and suppressor cells in mice expressing this allele.

The finding that the syngeneic killer differs from the allogeneic killer cell in expressing the  $\text{Lyt-1}^{-}\text{2}^{+}$  phenotype is somewhat surprising. However, other investigators have reported that anti-tumor syngeneic killer cells in C3H mice were predominantly of the  $\text{Lyt-1}^{-}\text{2}^{+}$  phenotype. These workers also noted the probable involvement of  $\text{Lyt-1}^{+}\text{2}^{+}$  cells in the syngeneic system.

In a recent report very applicable to this study, Mills and colleagues found that in DBA/2 mice, syngeneic tumor killers directed towards P815 tumor cells expressed  $\text{Lyt-1}$  antigen.(130). They however did not look at  $\text{Lyt-2}$  antigen expression on these cells. Therefore, as far as expression of  $\text{Lyt-1}$  on syngeneic killers goes, that study and the one reported here seem to be in conflict. One reason for this may be the different methods of generation of cytotoxic cells to P815. Syngeneic cytotoxic cells in the Mills study were generated in vitro (in the presence of IL-2) from P815 tumor bearing DBA/2 mice. Therefore, they were probably generating secondary cytotoxic killers as opposed to primary cytotoxic cells in this investigation. Thus, there may be a difference in the  $\text{Lyt-1}$  antigen expression on primary vs secondary syngeneic killers in the DBA/2-P815 system. This of course leads to the recent findings that all T cells probably express  $\text{Lyt-1}$  and  $\text{Lyt-2}$ , although in varying amounts (131).

Therefore, it is impossible to conclude at this time that the syngeneic killer cell is  $\text{Lyt-1}^{-}\text{2}^{+}$ , since it may only be quantitative differences between it and the allogeneic killer in expression of the  $\text{Lyt-1}$  alloantigen which appear to render them as distinct populations.

The observation made here that anti-Lyt-1 antiserum effectively abrogated suppressor cell function, in the in vitro system used, is explicable in at least two possible ways. First, it has been reported previously that suppressor cells which lower DTH reactions in mice of the  $\text{Lyt}^a$  allele are of the  $\text{Lyt-1}^{+2-}$  phenotype (120). Second, the cell that was isolated from immune spleens may not be the effector cell for suppression but may induce the suppressors in culture. Evidence for a mechanism such as this has been presented for both humoral and cell-mediated responses (42-44, 132,133). In the model presented by Germain, Benacaraf and colleagues (22,23,44,60,134-136), the first cell in immune suppression is a  $\text{Lyt-1}^{+}$ ,  $\text{I-J}^{+}$ , antigen-binding "inducer" of suppression. This cell may "induce", in a cascade-like mechanism, other T cells to actively suppress in the same system. The 5-day culture period of the assay here might allow the induction of suppressors in situ under the influence of this  $\text{Lyt-1}^{+2-}$  cell. At present, it can only be concluded that in a population of suppressor cells, or their precursors, an  $\text{Lyt-1}^{+2-}$  cell is essential for the suppressive effect to the observed in in vitro cytotoxic assays.

## CHAPTER IV

IN VITRO AND IN VIVO EFFECTS OF SYNGENEIC  
AND ALLOGENEIC ANTISERA RAISED TO TUMOR-  
SPECIFIC SUPPRESSOR FACTOR FROM DBA/2 MICE



## CHAPTER IV

Introduction

It is now recognized that T lymphocytes are made up of distinct subsets which interact with each other as well as B lymphocytes and macrophages to both amplify and inhibit a variety of immunological responses. Thus T cells have demonstrated enhancing effects on the immune response by way of T helper cells (2-10). T cells have also been found to inhibit the immune response by way of T suppressor cells (11-17,19-44,65).

In many of the investigations concerning antigen-specific regulatory T cells, it has been shown that soluble factors derived from these cells can apparently duplicate their functions (14,59-61,67-74,76,77,82,137-139). Little is known of these factors but, as indicated in Chapter II, what is known shows they exhibit certain physical, chemical and antigenic properties in common. They are proteins with specific antigen binding potential, have molecular weights of about 50,000, have determinants which are coded by the Ir region, lack constant region immunoglobulin determinants, but may carry variable (idiotypic) determinants of the  $I_g$  heavy chain. All this leads to the intriguing possibility that these "factors" may in fact be the antigen receptors of these specific regulatory T cells.

Yamauchi et al (125) have recently shown that injection of soluble S1509a tumor extracts into syngeneic A/J mice activates, in the spleen tumor-specific T suppressor cells. This has recently been extended to the syngeneic P815 tumor system in DBA/2J mice (Chapter III, 13). In this chapter, it is shown that by passing extracts of this suppressive

population over P815 membrane - Sepharose columns, P815-specific suppressor factor can be eluted.

This P815-specific suppressor factor was then injected into syngeneic and allogeneic groups of mice. The hope being that in the syngeneic mice the unique determinants (idiotypic?), found in or near the antigen binding site of the P815-specific suppressor factor, would elicit an antibody response in at least some of the animals. While both these and the constant determinants on the suppressor factor might induce an antibody response in the allogeneic mice. Both groups of mice produced antisera which was capable of reacting in vitro specifically with the P815-specific suppressor factor, and with complement was able to abrogate the function of P815-specific T suppressor cells, but was unable to effect the action of P815-specific T cytotoxic effector cells. The antisera produced in syngeneic mice was also shown to be effective in vivo in slowing P815-tumor growth and prolonging survival times of DBA/2 mice injected with this tumor.

## Materials and Methods

### Mice and Tumors.

Female DBA/2J and C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Except for the preparation of anti-suppressor factor antisera, all mice were used between the ages of 2 and 5 months of age. The tumor lines used were the P815 mastocytoma and L1210 leukemia, both syngeneic for DBA/2J mice. They have both been maintained and transplanted as ascites tumors in DBA/2J mice (11), or as frozen cultures maintained in liquid nitrogen in this lab for the past 6 years. Balent1 tumor, syngeneic to Balb/c mice, was also used and maintained as above.

### Cells.

This has been described in detail in Chapter III.

### Culture system for generation of cytotoxic effector cells.

The method by which a primary in vitro cytotoxic response to syngeneic tumor cells can be generated has been described previously (123). Briefly, specific cytotoxicity against either P815 or L1210 was generated in vitro by incubating  $5 \times 10^6$  spleen cells with  $5 \times 10^5$  mitomycin treated tumor cells in Linbro multiwell plates containing 24 flat bottom wells, at  $37^\circ\text{C}$  in a humidified incubator with 5%  $\text{CO}_2$  for 5 days. The total volume of each culture well was made up 2.5 ml with complete 1640 medium. After incubation, cells were harvested, counted, resuspended in complete 1640 medium at a concentration

of  $10^7$ /ml and titrated in quadruplicate with doubling dilutions starting at an effector:target ratio of 100:1 in a standard 18 h  $^{51}\text{Cr}$  release assay which has been described in previous chapters (II and III). The specific effector cells in this syngeneic tumor system have previously been shown to be T lymphocytes (11,13).

#### Generation of suppressor cells.

The method, for generation of tumor-specific T suppressor cells, involved in the regulation of cytotoxic response of DBA/2J mice to the syngeneic P815 tumor, has been described previously (Chapter III). Briefly, suppressor cells used in this study were generated by the intraperitoneal injection of soluble membrane extracts of P815 cells. Suppressor cells were readily induced in the spleens of DBA/2J mice by intraperitoneal injection of 150  $\mu\text{g}$  of protein per animal 4-5 days before sacrifice.

Because the spleens of these animals contained both antigen-specific and non-specific suppressor cells (13) it was necessary to separate the two populations. Spleen cells were passed through nylon wool columns by a modified method of Julius *et al.* (126). The method is basically that described before (Chapter III, 13) with a few modifications outlined below. In short, the cells, after application to the nylon wool column, were incubated for 60 min at room temperature ( $20^\circ\text{C}$ ) and then eluted with warmed  $37^\circ\text{C}$  complete 1640 medium. Approximately 15% of the cells were recovered by this elution process. The properties of the eluted cells were characteristic of T cell enriched populations in that

they were >95% sensitive to anti-Thy 1 serum plus complement and contained very few phagocytic cells (<1.0%). This population was antigen-specific in its suppressive activity, and has been found repeatedly to contain cells, which under the conditions used here, were capable of suppressing the in vitro primary cytotoxic response 30-70%.

#### Preparation of suppressor factor.

Antigen-specific T suppressor cells, obtained from spleens of DBA/2J mice injected intraperitoneally 4-5 days previously with P815 tumor membrane fragments were used for the preparation of P815 antigen-specific suppressor factor (P815-SF). Control factor preparations were made from normal DBA/2 splenocytes, as well as from mice injected with Balent1 tumor membrane fragments (this preparation was termed Balent1-"SF" even though it had no demonstratable effect, suppressive or otherwise). The splenocytes were sonicated for 3 one minute bursts (Biosonic 80, 60 watt setting) at 0°C. This material was then centrifuged for 30 min at 15,000 x G at 0°C. The supernatant was then taken and mixed with normal DBA/2J membrane fragments linked to Sepharose 4B. This step was taken to ensure that any material which non-specifically bound to DBA/2J membrane would be removed here. After 60 min at 0°C any non-bound material was eluted with ice cold phosphate buffered saline (PBS). This material was then mixed with rabbit anti-mouse immunoglobulin linked to Sepharose 4B. This was to remove any anti-body molecules which

Preparation of P815 Tumor-Specific  
Suppressor Factor (P815-SF)

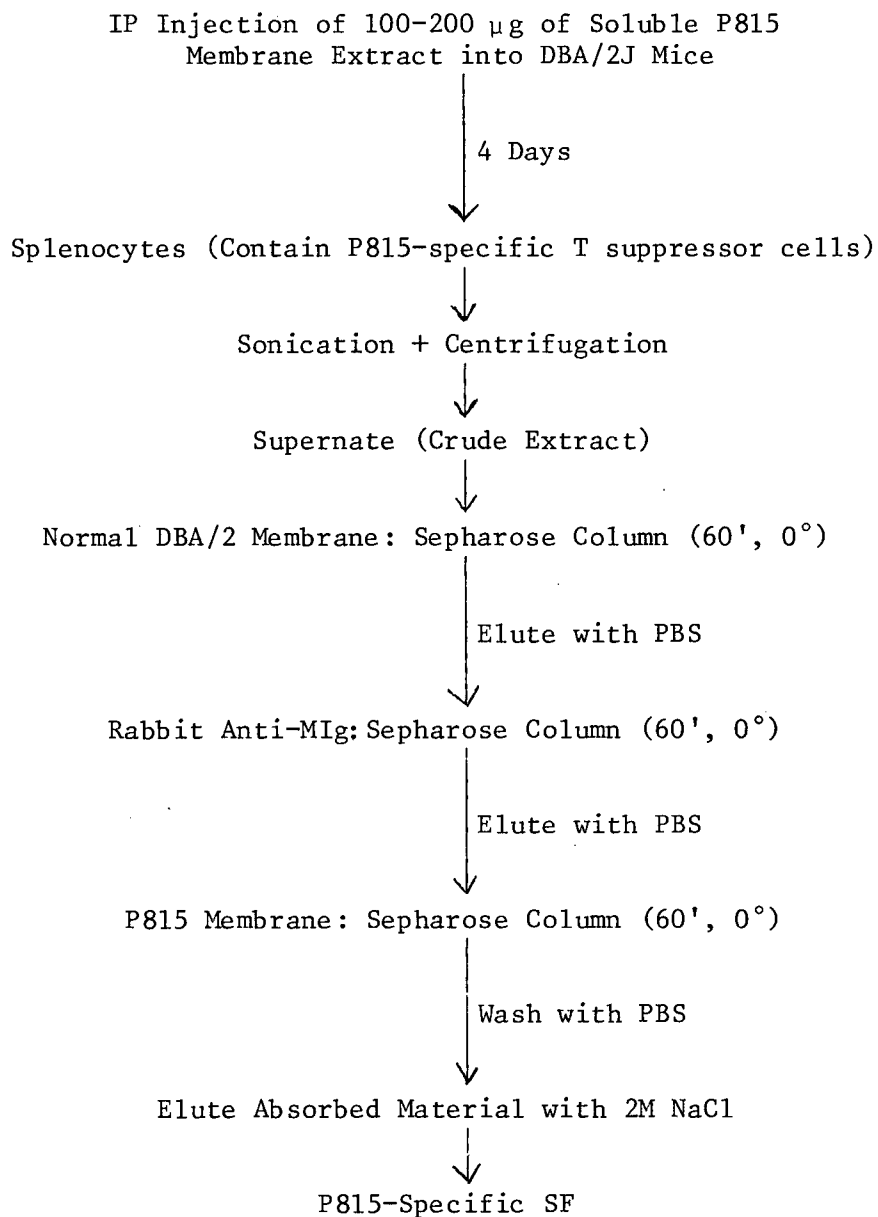


Figure 15. Flow diagram illustrating the preparation of P815 tumor-specific suppressor factor (P815-SF)

might bind in the following step. The capacity of the anti-mouse immunoglobulin column was sufficient to remove at least twice the amount of Ig found in the extracts. After 60 min at 0°C any non-bound material was eluted with ice cold PBS. This material was then mixed with P815 membranes linked to Sepharose 4B. After 60 min at 0°C the beads were washed thoroughly with ice cold PBS. Bound material was eluted under mildly dissociating conditions with 2M NaCl. The absorbance at 280 nm of this material was in all cases <0.01; therefore, it has been difficult to quantify it other than by its biological properties. It was filter sterilized and stored at -70°C. This material which was eluted from the P815 column was then titrated for suppressive activity. Concentration when mentioned is quoted as spleen equivalents based on the volume of original spleen homogenates corrected for the change in volume following passage through the columns. Values for the eluted fractions are based on the assumption that all the activity was recovered. All the above-mentioned columns were prepared as described previously (65,140) by the cyanogen bromide method (141). The preparation of suppressor factor is illustrated in Figure 15.

#### Assay for suppressor cells and suppressor factors.

The method used to assay for suppressor cells has been described in detail elsewhere (123). Briefly,  $5 \times 10^6$  normal spleen cells were cocultured with  $5 \times 10^6$  (or fewer in some cases) suppressor cell populations in the presence of  $5 \times 10^5$  mitomycin

C treated tumor cells, in 24 well Linbro trays in 2.5 ml of complete 1640 medium. Cells were cultured for 5 days before being assayed for cells cytotoxic to the tumor. Controls in these studies constituted the use of  $5 \times 10^6$  normal spleen cells plus  $5 \times 10^6$  normal spleen cells or spleen cells which had been treated in a manner analogous to the suppressor population. Assay for the splenic suppressor factor activity involved incubation of  $5 \times 10^6$  normal spleen cells with various concentrations of the factor preparation, under the same conditions as outlined above. All experiments reported herein, using these assays were repeated at least three times on separate occasions (therefore separate preparations of both suppressor cells and factor).

Preparation of antisera against P815-specific suppressor factor.

The anti-P815-specific suppressor factor antisera (anti-P815-SF antisera) used in this study was prepared by immunizing syngeneic DBA/2J mice and allogeneic C57BL/6 mice repeatedly with P815-specific immunoadsorbent eluted suppressor factor (see above). Control material (Balent1-"SF") was prepared in parallel from DBA/2J mice injected 4-5 days previously with Balent1 tumor membrane extracts. This material was injected into control groups of mice from the same batch. Animals were injected subcutaneously every 2 weeks with 0.1 ml (0.036 spleen equivalent) of either P815-specific suppressor factor or control Balent1-"suppressor factor" in complete Freund's adjuvant. After



4 months, the mice were bled from the retroorbital sinus once a month (one week after the last immunization) while maintaining the immunization schedule. The resulting antisera was inactivated at 56°C for 30 min, then tested for its ability to abrogate in the presence of complement the function of P815 specific T suppressor cells. In all experiments reported here the antisera from the mice within each group were pooled before testing. Each of the four groups contained at least 15 mice each.

Anti-P815-SF antisera killing of cells.

The cells tested for their expression of P815-specific suppressor factor determinants in this study were: syngeneic P815 tumor-specific suppressor T cells and syngeneic P815 tumor-cytotoxic T cells. In preliminary tests the two anti-P815-SF antisera pools were found to be effective, in conjunction with rabbit complement (Rabbit Low Tox Complement, Cedar Lane Labs, Hornby, Ontario), in eliminating suppressor cell function, at dilutions of up to 1:40. In experiments run here all the antisera was used at a final concentration of 1:10, in the presence of a 1:9 final concentration of complement. At this concentration no anti-normal DBA/2J or anti-tumor activity was detected in the anti-P815-SF antisera.

In experiments testing for the presence of P815-specific suppressor factor determinants on suppressor cells,  $2 \times 10^7$  splenic T suppressor cells, prepared from DBA/2J mice injected with tumor membrane extracts and  $2 \times 10^7$  splenocytes from normal

DBA/2 controls, were suspended in 0.40 ml of a 1:10 dilution of antisera and incubated for 45 min at room temperature. Then 50  $\mu$ l of neat rabbit complement was added and incubation was continued for another 45 min. Appropriate controls of complement and medium only were run concurrently. Following incubation cells were washed twice in complete 1640 medium, and counted by trypan blue exclusion. In no instance were viable counts in cell suspensions containing the anti-P815-SF antisera significantly different from those of the controls, and in no instance were counts lower than 90% of the number of cells before treatment. These cells were subsequently used in suppressor cell assays.

In experiments testing for the presence of P815-specific suppressor factor determinants on cytotoxic cells, the protocol was as described above except  $1.6 \times 10^7$  cells, harvested from 5 day in vitro cultures with mitomycin C treated tumor cells, were treated and subsequently tested in the cytotoxicity assay. Since this experiment essentially resulted in negative results, cytotoxic cells were also treated with monoclonal anti-Thy 1 antisera (kindly supplied by Dr. H.-S. Teh, U.B.C., and used at a dilution of 1:100) plus complement.

#### Anti-SF antisera absorption of suppressor factor activity.

The ability of anti-P815-SF antisera to remove the suppressive activity from P815-specific suppressor factor preparations was tested by an immunoadsorbent assay similar to the one described previously (Chapter II, 65). In short, P815-specific suppressor

factor preparations or normal control material prepared in parallel were mixed with either the anti-P815-SF antisera or the control anti-Balenti-"SF" antisera, and incubated for 120 min at 0°C. These materials were then passed through immuno-adsorbent Sepharose 4B columns to which rabbit anti-mouse immunoglobulin had been attached by cyanogen bromide treatment. The capacity of the column was sufficient to remove at least twice the amount of mouse immunoglobulin added to the suppressor factor preparations. The material eluted from the columns was titrated for suppressive activity over dilutions ranging from 1:25 to 1:300 (these dilutions of eluted material represent between 0.036 and 0.0033 spleen equivalents).

#### ELISA assay.

This test was carried out as described before.(142). Briefly, 0.2 ml of antigen (e.g. P815-SF in Fig 18) in pH 9.6 carbonate buffer was attached to substrate microtiter plates (Cooke Engineering Co., Alexandria, Va., No. 1-220-295) for 18 hr at 4°C. After washing with PBS-tween buffer, antisera to be tested for their activity against the coated antigens were added to the wells in 0.2 ml aliquots. Following incubation for 2 hr at room temperature and subsequent washing, the developing alkaline phosphatase-linked rabbit anti-mouse Ig (RaMI<sub>g</sub>) or goat anti-rabbit (GoRI<sub>g</sub>), at a dilution of 1:600 or 1:400 respectively, was added in 0.2 ml aliquot. After a further 2 h incubation and final washing with buffer, 0.2 ml of the enzyme substrate solution (Sigma-104-105, p-nitrophenyl phosphate

disodium) was added to each well and the enzyme substrate reaction was allowed to continue while color developed in the wells. The color change was followed on a Titertek Muliskan (Flow Laboratories). All tests were done at least in triplicate. Controls of normal mouse serum (NMS), normal rabbit serum (NRS) or antigen alone with no added serum were always run and the appropriate control response was subtracted to get the specific response.

#### Antisera plus complement killing of P815 tumor cells.

P815 tumor cells were labeled with  $^{51}\text{Cr}$  as described previously (Chapter II). The  $^{51}\text{Cr}$  labeled P815 tumor cells were resuspended at  $2 \times 10^6/\text{ml}$  in complete medium. 100  $\mu\text{l}$  ( $2 \times 10^5$ ) of these cells were dispensed into the wells of multi-dish microculture plates. 50  $\mu\text{l}$  of antisera plus 50  $\mu\text{l}$  of 1:2 dilution of rabbit complement was then added to each well. Appropriate controls of complement and medium only were run concurrently. They were then incubated for 90 minutes at room temperature. 0.10 ml of the supernatant was removed, and its radioactivity was measured on a gamma counter.

#### In vivo effects of anti-P815-SF antisera.

The in vivo experiments of anti-P815-SF antisera were carried out to determine their effect on P815 tumor growth in DBA/2 mice. Two days prior to injection of P815 tumor cells 50  $\mu\text{l}$  of anti-P815-SF antisera, anti-Balenti-"SF" antisera, or PBS were injected i.v. into DBA/2 mice. Two days later  $2 \times 10^3$  P815 tumor

cells were injected s.c. into these mice. Tumor growth was followed by two dimensional measurements with calipers. There were 7 mice/groups, and these experiments were carried out on three separate occasions. Therefore, a total of 21 mice in each of the four groups. The combined results are reported here.

#### Analysis of Data.

On all occasions, experiments were repeated a minimum of three times. Within each experiment, all treatment groups were tested for cytotoxicity in quadruplicate over an effector:target ratio range of 100:1 to 12.5:1 in the  $^{51}\text{Cr}$  release assay. Quantification of cytotoxicity in lytic units was calculated as described previously (Chapters II and III) and total lytic units from recovered cells after in vitro culture is recorded in the figure or table legends. Statistical analysis of data from repeated experiments was done by Students' t-test. These results are shown in tables where appropriate.

## Results

Since it had been found (Chapter III, 13, 125) that intraperitoneal injection of solubilized tumor membrane antigens induce, in the spleen, T suppressor cells specific for that tumor, an attempt was made to prepare from these cells relatively pure specific suppressor factor. The relative purification of the P815-specific suppressor factor was achieved by passing crude spleen extracts, from DBA/2J mice previously injected intraperitoneally with solubilized P815 tumor extract, over immunoadsorbent columns made up of membrane extracts of P815 cells, and subsequently eluting from the column the P815-specific suppressor factor with 2M NaCl.

Figure 16a shows, that when added to in vitro cultures, the P815-specific suppressor factor significantly inhibits the generation of cytotoxic cells to P815 tumor cells when compared to untreated control cultures. The specificity of the P815-specific suppressor factor is shown by its inability to affect the generation of cytotoxic cells to L1210, another tumor syngeneic to DBA/2J mice (Fig. 16b).

The P815-specific suppressor factor was found to be relatively stable when stored at  $-70^{\circ}\text{C}$ , and was active at very high dilutions. Figure 17 shows a typical titration curve for this suppressor factor. While the suppressor factor was very inhibitory at high dilutions, at high concentrations it was not, in fact sometimes it was somewhat stimulatory. The reason for this is not understood, but this was a reproducible observation. The titration curve (Figure 17) may possibly be accounted for by the presence of P815-specific helper

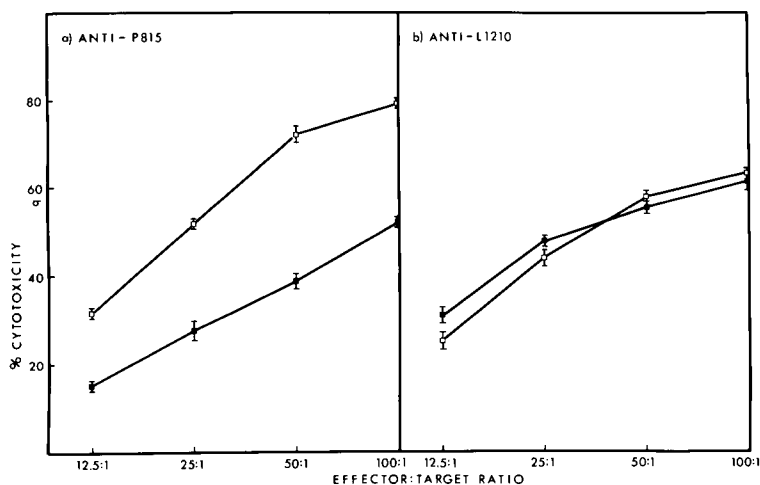


Figure 16. The ability of P815-specific suppressor factor to suppress the in vitro primary cytotoxic response of normal DBA/2J splenocytes to syngeneic mitomycin C treated P815 tumor cells.

(a) Anti-P815: the cytotoxic activity of cells primed in vitro with P815 cells on  $^{51}\text{Cr}$  labelled P815 cells in an 18 h assay.

(b) Anti-L1210: the cytotoxic activity of cells primed in vitro with L1210 cells on  $^{51}\text{Cr}$  labelled L1210 cells in an 18 h assay.

■—■, primary culture of normal DBA/2J splenocytes and 1/150 dilution (0.0071 spleen equivalent) of P815-specific suppressor factor. Total lytic units: (a) 7.35 (b) 29.30.

□—□, primary culture of normal DBA/2J splenocytes, no suppressor factor added. Total lytic units (a) 31.32 (b) 27.17

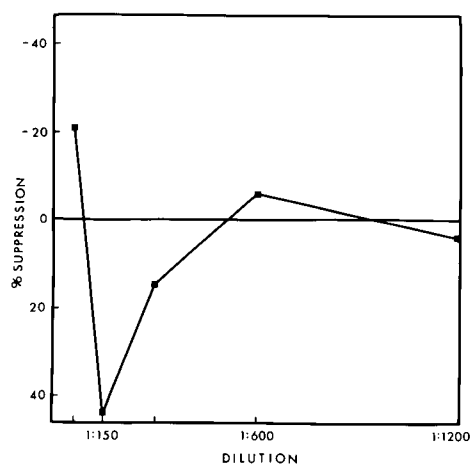


Figure 17. Titration of immunoadsorbent purified P815-specific suppressor factor. Cytotoxic values were calculated at an effector:target ratio of 50:1 and converted to % suppression by comparison with equivalent cultures incubated in the presence of a control suppressor factor isolated in an analogous fashion from L1210 primed mice.



factor which could co-purify with the suppressor material. However, other investigators, working with suppressor factor producing hybridomas have also found that high concentrations of hybridoma supernatants are sometimes much less suppressive than are higher dilutions (143), so this may not be the explanation. While the methods used for the purification of the P815-specific suppressor factor were carried out to maximize the purity of the factor, it is quite probable that the material eluted from P815 columns contained a number of contaminants. Because of the exceedingly small amounts of protein in our factor preparations ( $<0.01$  absorbance at 280 nm), at this time it is difficult to establish its degree of purity by conventional biochemical means. However, it appears that this material has properties analogous to those described previously (Chapter II, 65, 123) for a suppressor factor isolated by similar methods from thymocytes of P815-bearing mice (Chapter II, 65).

Taken together, these results show that a very inhibitory and highly specific suppressor factor can be prepared from spleens of DBA/2J mice previously injected intraperitoneally with soluble tumor membrane extracts.

Since we had a relatively pure, specific suppressor factor, a program was set up to immunize groups of both syngeneic DBA/2J and allogeneic C57BL/6 mice with P815-specific suppressor factor. The hope was that in the syngeneic mice the unique determinants (idiotypic?), found in or around the antigen binding site of the P815-specific suppressor factor, would elicit an antibody response in at least some of the animals. While both these and the constant

determinants on the suppressor factor might induce an antibody response in the allogeneic mice. Control groups of mice were injected with material (Balent1-"SF") prepared in an identical fashion (i.e. final dilution off of P815 membrane columns) from DBA/2J mice, injected intraperitoneally with Balent1 tumor extracts. All the results reported here are from antisera pooled within each of the four groups.

The fact that the DBA/2J and C57BL/6 antisera thus prepared contained anti-P815-SF activity was shown by their ability to absorb out the inhibitory activity of the P815-specific suppressor material. A representative set of data is shown in Table II. P815-specific suppressor factor was mixed with either the anti-P815-SF antisera or anti-Balent1-"SF" antisera, and subsequently absorbed on immuno-adsorbent columns containing anti-mouse immunoglobulin. The unattached eluted material was then tested for suppressive activity. While suppressive activity was still present in the control anti-Balent1-"SF" antisera treated eluates, the suppressive material treated with anti-P815-SF antisera lost its activity, thus showing that both DBA/2J anti-P815-SF antisera and C57BL/56 anti-P815-SF antisera recognize and can bind determinants expressed on the P815-specific suppressor factor. This experiment was run on three separate occasions and, the differences were all similar and significant in each case.

The ability of the anti-P815-SF antisera and not the control anti-Balent1-"SF" antisera to bind to determinants expressed on P815-SF is shown using a different method in Figure 18. The ELISA assay was used to show that both the DBA/2 and the C57BL/6 anti-P815-SF antisera reacted positively with P815-SF, while neither of

Table II. Anti-P815 antisera is able to react and bind to P815-specific suppressor factor. Suppressor factor preparations were tested after reaction with various mouse antisera and absorption on insolubilized rabbit anti-mouse Ig.

P815-Specific <sup>a</sup> SF added	P815-Specific SF <sup>b</sup> Absorbed With	% Cytotoxicity <sup>c</sup>		Total Lytic <sup>e</sup> Units	P <sup>f</sup>
		100:1 <sup>d</sup>	50:1 <sup>d</sup>		
+	DBA anti-P815-SF	53.4 ± 3.1	33.8 ± 2.6	36.4	N.S.
+	DBA anti-Balenti-"SF"	43.0 ± 3.1	23.5 ± 0.9	23.1	<.005
+	C57BL/6 anti-P815-SF	74.8 ± 5.8	35.3 ± 2.2	39.7	N.S.
+	C57BL/6 anti-Balenti- "SF"	49.1 ± 1.9	20.9 ± 1.1	25.8	<.005
+	-	41.1 ± 3.9	17.5 ± 1.4	21.7	<.005
-	-	56.9 ± 0.9	36.7 ± 1.3	39.6	

<sup>a</sup> SF added at the start of 5 day in vitro cultures for the generation of cytotoxic cells as described in Materials and Methods. SF was treated as outlined in next column prior to addition. A 1/150 dilution (0.0071 spleen equivalent) of SF was used.

<sup>b</sup> SF was mixed with the antisera then passed through an anti-mouse Ig column. Material which passed through was tested for suppressive ability. Control SF preparations were only passed through anti-mouse Ig column then tested.

<sup>c</sup> Cytotoxicity was tested by 18 h <sup>51</sup>Cr release assay. Numbers are % specific release ± S.E.M.

<sup>d</sup> Effector:Target ratio.

<sup>e</sup> One lytic unit was defined as the number of effector cells required to lyse 50% of 10<sup>4</sup> target cells. Total lytic units were the number of lytic units found in the recovered cell population.

<sup>f</sup> Data were analysed by Students' t-test. N.S. - not significant. P values of <.05 were considered significant.

Table III. Results of Students' t-test run on 3 separate experiments testing 3 individual bleeds of mice immunized with P815-SF or control Balent1-"SF". Differences between groups were calculated from data obtained in  $^{51}\text{Cr}$  release assay at effector-target ratios of 50:1 (levels at which killing is in the linear part of the curve). All groups were compared to the killing in cultures containing suppressor cells which had been treated only with C.

Treatment of Suppressor Cells	$p^a$		
	Expt. 1	Expt. 2	Expt. 3
DBA anti-P815-SF	<.025	<.005	<.005
DBA anti-Balent1-"SF"	N.S.	N.S.	N.S.
C57BL/6 anti-P815-SF	<.05	<.05	<.005
C57BL/6 anti-Balent1-"SF"	<.025	N.S.	N.S.

<sup>a</sup>P values of <.05 are considered significant.

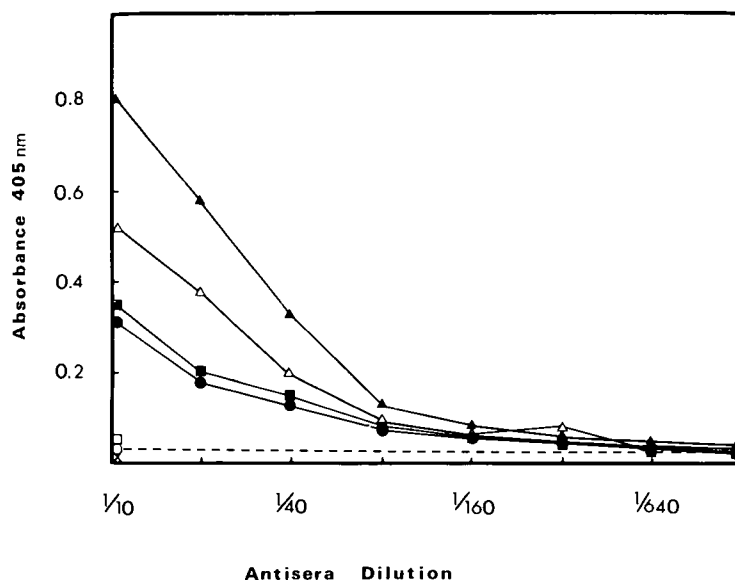


Figure 18. Characterization of P815-SF by ELISA assay. P815-SF (0.0023 spleen equivalent) was used as antigen in the ELISA. Various antisera were added to determine what characteristics were expressed on the P815-SF. ●—●, DBA/2 anti-P815-SF; ○—○, DBA/2 anti-Balenti-"SF"; ■—■, C57BL/6 anti-P815-SF, □—□, C57BL/6 anti-Balenti-"SF", ▲—▲, anti-H-2<sup>d</sup>; △—△, anti-Ia<sup>d</sup>; x—x, anti-MIg.

Incubation of plate after addition of enzyme substrate was for 45 minutes.

the anti-Balenti-"SF" antisera reacted above background levels. These results confirm the results of the absorption experiments (Table II). Another important feature to note is which of the other antisera preparations reacts with the P815-SF. While both anti-H2<sup>d</sup> and anti-Ia<sup>d</sup> antisera react with P815-SF, anti-mouse Ig antisera does not. This shows that P815-SF has determinants coded by the Ia region of the H-2 major histocompatibility complex, but does not have "common" mouse Ig constant region determinants. These properties are the same as those described previously for P815-specific suppressor factor isolated from thymocytes of P815 bearing mice (Chapter II, 65,21).

The possibility that the activity of the P815-specific T suppressor cells could be abrogated by anti-P815-SF antisera plus complement was investigated. Prior to addition to normal DBA/2J spleen cells plus mitomycin C treated P815 cells at the start of in vitro culture, the specific T suppressor cell population was treated with complement plus either anti-P815-SF antisera or anti-Balenti-"SF" antisera. Both of the anti-P815-SF antisera produced in the syngeneic and allogeneic mice were effective in eliminating the suppressive effect normally produced by these cells (Fig. 19). Again, as with the adsorption of the P815-specific suppressor factor, neither of the anti-Balenti-"SF" antisera controls had an effect. With all the bleeds tested the DBA/2J anti-P815-SF antisera and the C57BL/6 anti-P815-SF antisera were approximately equally effective (Table III). The data presented in Figure 19 is from experiment 2. Therefore, it appears that in this system the suppressor cell, its progenator,

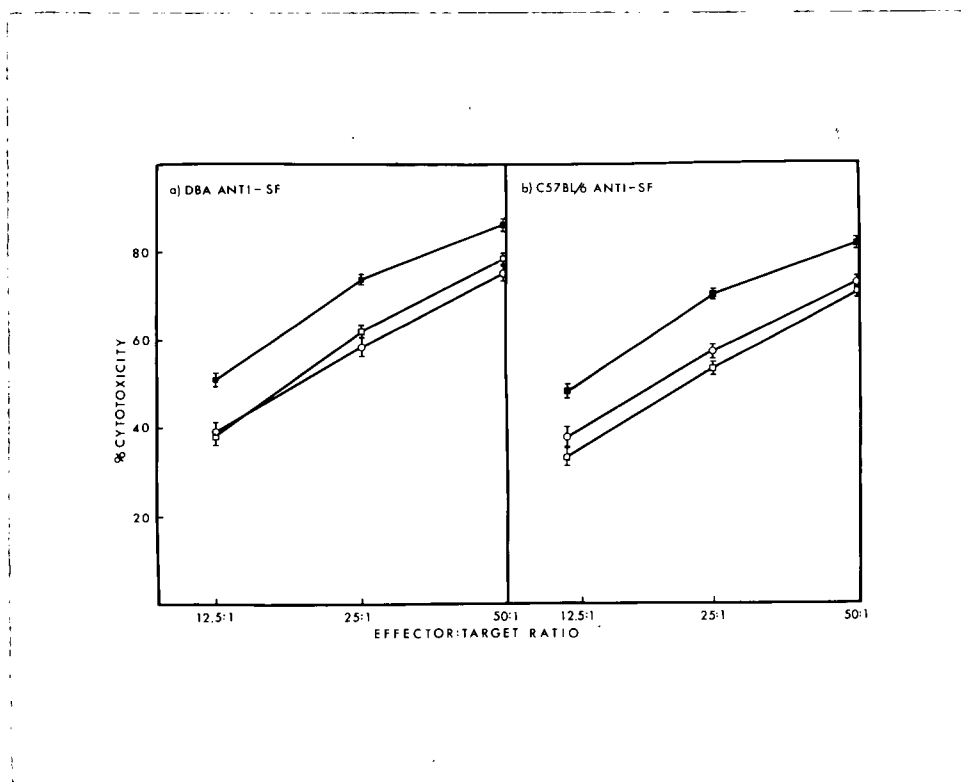


Figure 19. The ability of anti-P815-SF antisera plus complement to eliminate P815-specific suppressor cells.

(a) DBA/2J anti-SF:- the cytotoxic response of cells primed in vitro with P815 after treatment of the suppressive splenocytes with DBA/2 anti-SF antisera plus complement.

(b) C57BL/6 anti-SF:- the cytotoxic response of cells primed in vitro with P815 after treatment of the suppressive splenocytes with C57BL/6 anti-SF antisera plus complement.

■—■, primary culture of normal DBA/2J splenocytes plus anti-P815-SF and complement treated P815-specific suppressor splenocytes. Total lytic units (a) 101.02 (b) 90.13

□—□, primary culture of normal DBA/2J splenocytes plus anti-Balenti-SF and complement treated P815 specific suppressor splenocytes. Total lytic units (a) 63.19 (b) 52.25

○—○, primary culture of normal DBA/2J splenocytes plus complement only treated P815-specific suppressor splenocytes. Total lytic units (a) and (b) 57.54.

or a cell vital for its development, expresses on its surface, suppressor factor, or at least determinants common to it.

The possibility that in vitro generated syngeneic cells cytotoxic for P815 could also be killed by these antisera was also investigated. In vitro generated cytotoxic T cells specific for either P815 or L1210 were treated with complement plus either anti-P815-SF antisera or anti-Balenti-"SF" antisera. As can be seen in Table IV, neither the syngeneic DBA/2J anti-P815-SF antisera nor the allogeneic C57BL/6 anti-P815-SF antisera had any effect on the killing carried out by the cytotoxic T cells. Therefore, showing that in the syngeneic P815 tumor system of DBA/2J mice the specific cytotoxic T cells lack determinants in common with those found on either the specific T suppressor cells or the specific suppressor factor as detected by the anti-SF antisera. In repeats of these experiments, no significant differences were found between groups on any occasions.

Finally the in vivo effect of the anti-P815-SF antisera on P815 tumor growth in DBA/2 mice was assessed. Two days prior to injection with P815 tumor cells, DBA/2 mice were injected intravenously with 50  $\mu$ l of one of the four antisera preparations on PBS. Figure 20 shows the growth of the tumor after injection subcutaneously. As can be seen the DBA/2 anti-P815-SF antiserum greatly slowed the growth of the P815 tumor. The other antisera preparations had no effect on tumor growth. These results are also born-out in the improved survival time of animals injected with DBA/2 anti-P815-SF antisera (Table V). The mice injected with DBA/2 anti-P815-SF antisera had a 48% increased survival time compared to those injected with PBS



Table IV. Anti-P815-SF antisera plus complement (C') treatment has no effect on the ability of specific cytotoxic effector T cells to lyse the appropriate target.

Effectors Generated Against <sup>a</sup>	Cells Treated With <sup>b</sup>		% Cytotoxicity <sup>c</sup>		p <sup>e</sup>
	Antisera	C'	100:1 <sup>d</sup>	50:1 <sup>d</sup>	
P815	Anti-Thy 1	+	6.6 ± 3.3	2.5 ± 1.4	<.005
	DBA anti-P815-SF	+	40.5 ± 2.5	28.5 ± 4.7	N.S.
	DBA anti-Balenti-"SF"	+	36.9 ± 4.7	29.8 ± 3.0	N.S.
	C57BL/6 anti-P815-SF	+	45.9 ± 4.8	32.4 ± 3.5	N.S.
	C57BL/6 anti-Balenti-"SF"	+	45.0 ± 4.0	32.2 ± 3.8	N.S.
	-	+	44.0 ± 5.2	31.7 ± 3.1	
	-	-	45.2 ± 3.8	34.9 ± 2.3	
			25:1 <sup>d</sup>	12.5:1 <sup>d</sup>	
L1210	Anti-Thy 1	+	9.8 ± 2.7	1.2 ± 0.8	<.005
	DBA anti-P815-SF	+	40.5 ± 3.9	24.1 ± 1.6	N.S.
	DBA anti-Balenti-"SF"	+	42.7 ± 2.3	26.6 ± 2.1	N.S.
	C57BL/6 anti-P815-SF	+	39.2 ± 4.1	27.7 ± 2.7	N.S.
	C57BL/6 anti-Balenti-"SF"	+	47.9 ± 3.2	21.5 ± 3.2	N.S.
	-	+	39.2 ± 3.8	27.2 ± 1.0	
	-	-	39.8 ± 0.8	26.2 ± 2.2	

<sup>a</sup>Effectors were generated *in vitro* by mixing DBA/2J spleen cells with the appropriate mitomycin C treated tumor cells and incubating them for 5 days as described in the Materials and Methods.

<sup>b</sup>The effector cells were treated with antisera plus complement then washed and tested for their ability to lyse the appropriate <sup>51</sup>Cr labelled targets as described in Materials and Methods. Control effector populations were treated with only complement or media alone.

<sup>c</sup>Cytotoxicity was tested by 18 h <sup>51</sup>Cr release assay. Numbers are % specific release ± S.E.M.

<sup>d</sup>Effector:Target ratio.

<sup>e</sup>Data were analysed by Students t-test. N.S. - not significant.

In three experiments, no significant differences were found between any of the anti-SF groups.

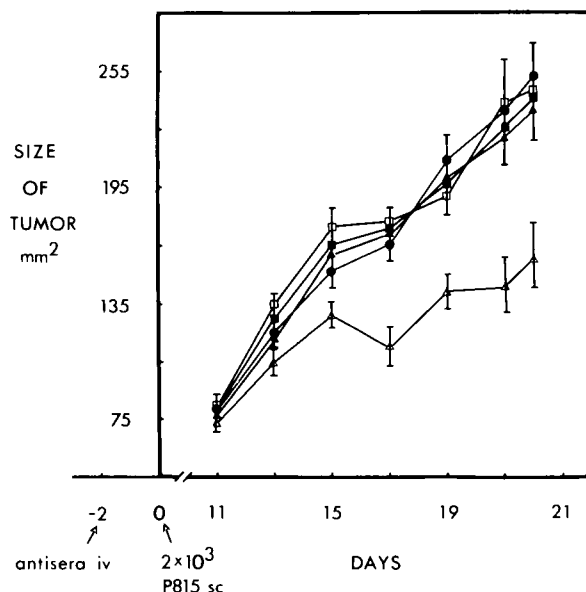


Figure 20. Effect of anti-P815-SF antisera in vivo on P815 tumor growth<sub>3</sub> in syngeneic DBA/2 mice. Mice were injected with  $2 \times 10^3$  P815 subcutaneously on day 0. Two days prior 50  $\mu$ l of antisera had been injected intravenously. Tumor size was measured in two dimensions with calipers. This figure shows the combined results of three separate experiments with a total of 21 mice/group.  $\Delta$ — $\Delta$ , DBA/2 anti-P815-SF;  $\square$ — $\square$ , C57BL/6 anti-P815-SF;  $\blacktriangle$ — $\blacktriangle$ , DBA/2 anti-Balenti-"SF";  $\blacksquare$ — $\blacksquare$ , C57BL/6 anti-Balenti-"SF";  $\bullet$ — $\bullet$ , PBS.

Table V. Effect of anti-P815-SF antisera in vivo on survival time of DBA/2 mice after P815 tumor injection<sup>a</sup>.

DBA/2 mice injected <sup>b</sup> i.v. with	mean survival time (days) <sup>c</sup>			
	10	20	30	40
DBA/2 anti-P815-SF antisera	38.1 ± 4.4 p<.005			
DBA/2 anti-Balenti-"SF" antisera	29.0 ± 2.3 N.S.			
C57BL/6 anti-P815-SF antisera	27.2 ± 3.5 N.S.			
C57BL/6 anti-Balenti-"SF" antisera	29.5 ± 2.4 N.S.			
PBS	25.7 ± 2.5			

<sup>a</sup>Three separate experiments were run and the results were combined for this table.

<sup>b</sup>DBA/2 mice were injected i.v. with 50 µl of antisera two days prior to P815 tumor challenge. 2 x 10<sup>3</sup> P815 tumor cells were injected s.c. on day zero.

<sup>c</sup>The mean survival time of individual groups (21 mice/group) of mice after injection of 2 x 10<sup>3</sup> P815 tumor cells s.c.

alone. Therefore, even though both DBA anti-P815-SF antisera and C57BL/6 anti-P815-SF antisera were reactive in vitro only the syngeneic DBA/2 preparation shows any effect in vivo.

Experiments were also carried out in vivo with the anti-P815-SF antisera to assess its effect on L1210 tumor growth in DBA/2 mice. The results shown in Figure 21 and Table VI show that neither the syngeneic DBA/2 anti-P815-SF nor the C57BL/6 anti-P815-SF antisera had a statistically significant effect on L1210 tumor growth in vivo.

Since the preparation of the P815-SF used to induce the anti-P815-SF antisera entailed a final elution step off a P815 membrane: Sepharose column, there is a possibility that the P815-SF material could also contain small amounts of P815 antigen which was shed off the column along with the bound suppressor factor. It is therefore possible that the antisera may contain anti-P815 antigen activity, which could interfere with the interpretation of some of the results reported here. Two different experiments were set up to explore this possibility.

The first is shown in Figure 22. Here the two anti-P815-SF antisera were tested for their ability to lyse <sup>51</sup>Cr labeled P815 tumor cells in the presence of complement. As can be seen neither the DBA/2 anti-P815-SF antisera nor the C57BL/6 anti-P815-SF antisera had any anti-P815 lytic ability.

The second experiment, to determine if the two anti-P815-SF antisera contain any anti-P815 activity, was to react these antisera with P815 membrane extracts in an ELISA assay. It can be seen in Figure 23 that both DBA/2 and C57BL/6 anti-P815-SF antiserum contain

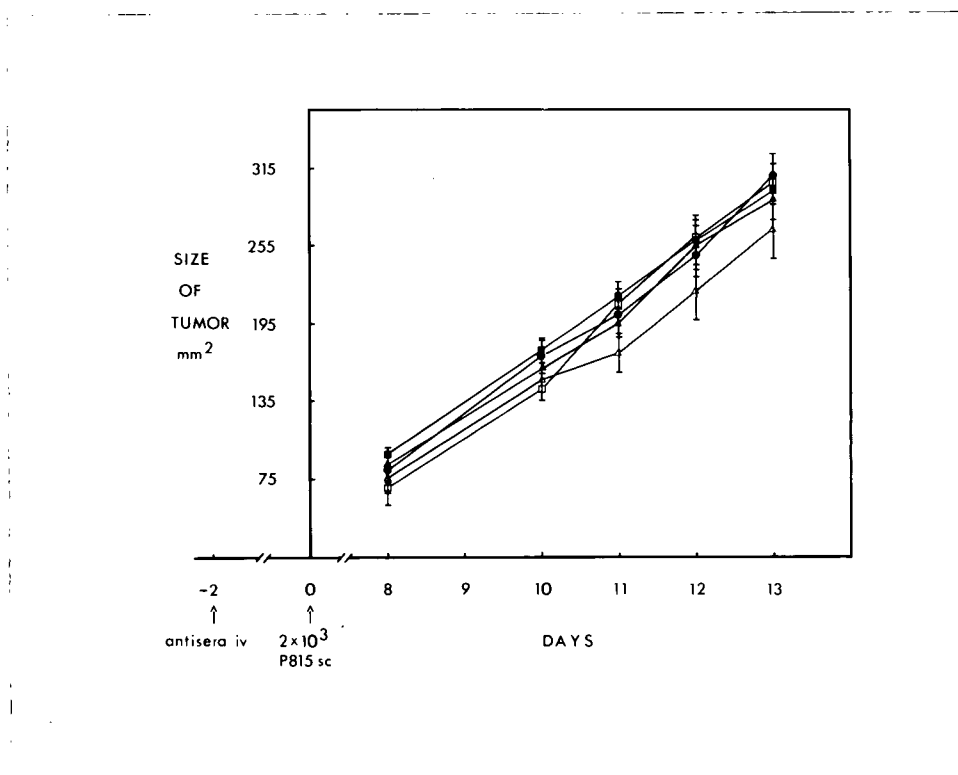


Figure 21. Effect of anti-P815-SF antisera in vivo on L1210 tumor growth<sub>3</sub> in syngeneic DBA/2 mice. Mice were injected with  $2 \times 10^3$  L1210 subcutaneously on day 0. Two days prior 50  $\mu$ l of antisera had been injected intravenously. Tumor size was measured in two dimensions with calipers. This figure shows the combined results of two separate experiments with a total of 18 mice/group.  $\Delta$ — $\Delta$ , DBA/2 anti-P815-SF;  $\square$ — $\square$ , C57BL/6 anti-P815-SF;  $\blacktriangle$ — $\blacktriangle$ , DBA/2 anti-Balenti-"SF";  $\blacksquare$ — $\blacksquare$ , C57BL/6 anti-Balenti-"SF";  $\bullet$ — $\bullet$ , PBS.

Table VI. Effect of anti-P815-SF antisera in vivo on survival time of DBA/2 mice after L1210 tumor injection<sup>a</sup>.

DBA/2 mice injected <sup>b</sup> i.v. with	mean survival time (days) <sup>c</sup>			
	5	10	15	20
DBA/2 anti-P815-SF antisera	17.4 ± 1.9	N.S.		
DBA/2 anti-Balenti-"SF" antisera	16.7 ± 1.3	N.S.		
C57BL/6 anti-P815-SF antisera	15.7 ± 1.5	N.S.		
C57BL/6 anti-Balenti-"SF" antisera	16.5 ± 2.2	N.S.		
PBS	16.7 ± 2.1			

<sup>a</sup>Two separate experiments were run and the results were combined for this table.

<sup>b</sup>DBA/2 mice were injected i.v. with 50 µl of antisera two days prior to L1210 tumor challenge.  $2 \times 10^3$  L1210 tumor cells were injected s.c. on day zero.

<sup>c</sup>The mean survival time of individual groups (18 mice/group) of mice after injection of  $2 \times 10^3$  L1210 tumor cells s.c.

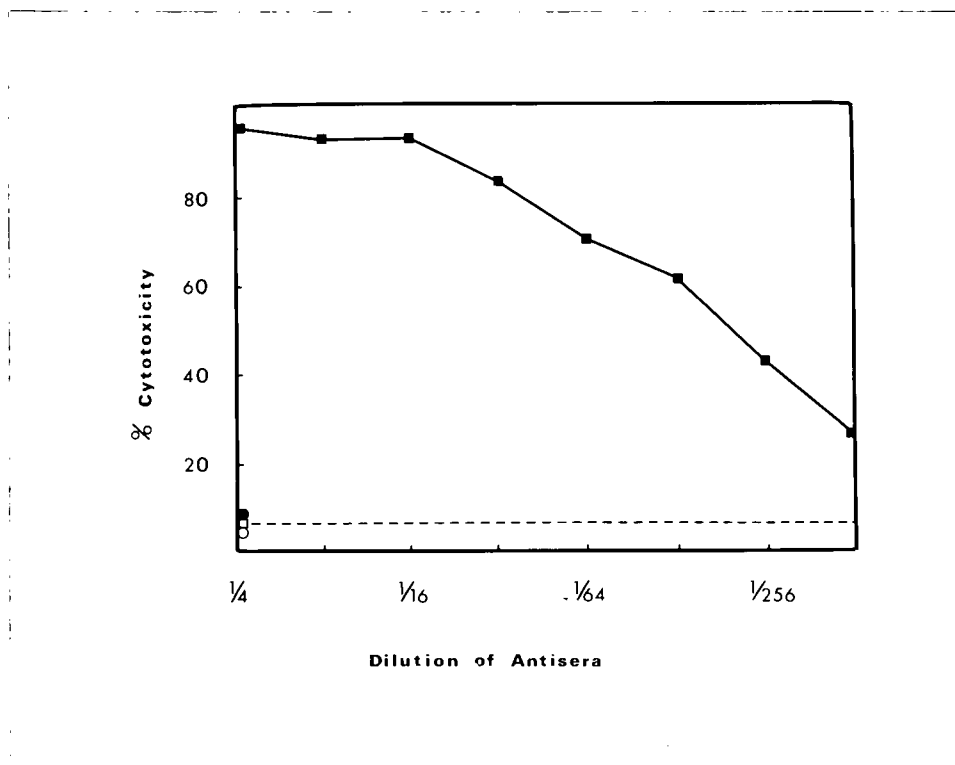


Figure 22. The inability of anti-P815-SF antisera, in the presence of complement, to lyse  $^{51}\text{Cr}$  labeled P815 tumor cells.  $^{51}\text{Cr}$  labeled P815 tumor cells were incubated with various dilutions of antisera plus complement. ●—●, DBA/2 anti-P815-SF; ○—○, C57BL/6 anti-P815-SF; ■—■, anti-H-2<sup>d</sup>; □—□, anti-Ia<sup>d</sup>.

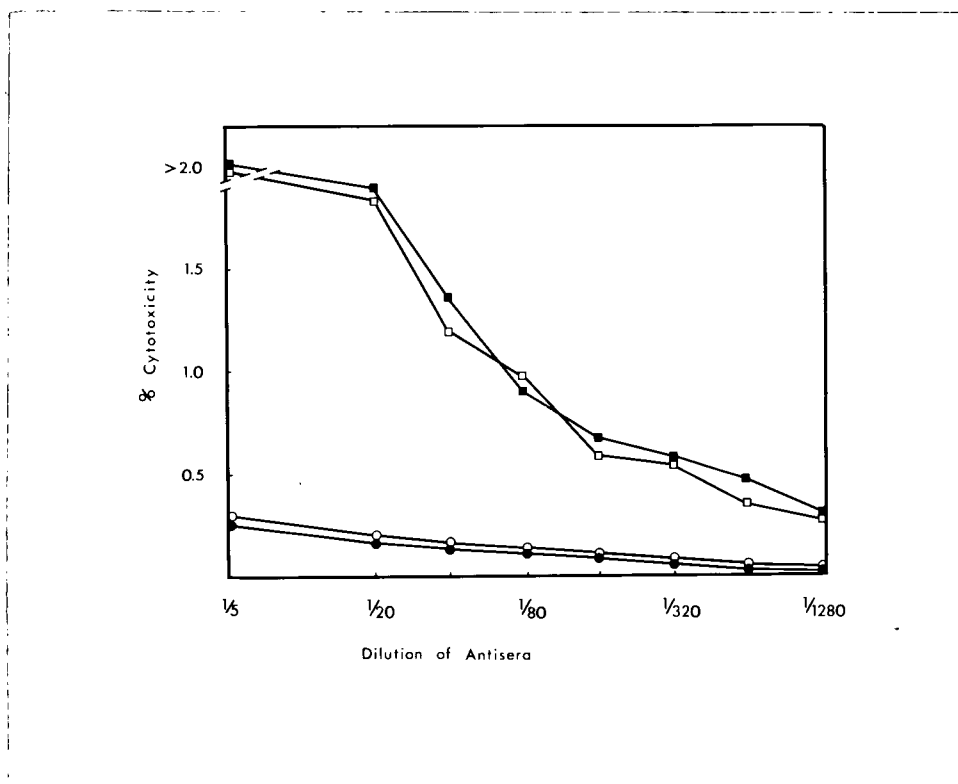


Figure 23. Does anti-SF antisera have any activity directed towards P815 tumor membrane determinants? Crude P815 membrane extract was used as antigen in the ELISA assay to determine if anti-SF antisera has any activity directed against P815. ●—●, DBA/2 anti-P815-SF, ○—○, C57BL/6 anti-P815-SF, ■—■, rabbit anti-P815; □—□, rabbit anti-DBA/2. Incubation of plates after addition of enzyme substrate was for 90 minutes.



only a very small amount of anti-P815 reactivity. It should be noted that the ELISA assay is a very sensitive method of detecting antibody reactivity, with only very small amounts (as little as 50 ng/ml) of specific antibody necessary to show a positive reaction in a very short time (142). Therefore the amount of anti-P815 reactivity found in the anti-P815-SF antisera preparations is a very small percentage of the total reactivity and probably plays no role in any of the experiments reported here, especially the ones mediated through complement.

## Discussion

The results presented previously (Chapter II) show that intraperitoneal injection of solubilized P815 tumor membrane antigen into syngeneic DBA/2J mice induces, in the spleen, T suppressor cells specific for P815. It was shown in this chapter that a very inhibitory and relatively purified P815-specific suppressor factor could be prepared from these suppressive spleen cells. This P815-specific suppressor factor was injected into syngeneic DBA/2J and allogeneic C57BL/6 mice and the resulting antisera was shown to (a) interact with P815-specific suppressor factor by absorption studies and ELISA assays, (b) interact with P815-specific T suppressor cells by complement mediated killing studies, and (c) not to interact with P815-specific T cytotoxic cells by complement mediated killing studies, while (d) only the syngeneic antisera was shown to be effective in vivo.

The results shown in Figure 16 indicate that a suppressor factor extracted from a suppressive cell population can duplicate the biological function of these cells. In this case both the T suppressor cell and the suppressor factor presumed derived from it are able to specifically suppress the primary in vitro generation of cytotoxic T cells to P815 (8, Chapter II). These results plus those in Figure 18 show that this suppressor factor has properties analogous to those described previously for a suppressor factor isolated by similar methods from thymocytes of P815-bearing DBA/2 mice (9,21,65,123, Chapter II).

It was then possible to use the P815-suppressor factor to prepare anti-P815-SF antisera. This was done by injecting both syngeneic

DBA/2 and allogeneic C57BL/6 mice with P815-SF. As Table II and Figures 18 and 19 show the resulting anti-P815-SF antisera could react with determinants expressed on the suppressor factor and the suppressor cell it is presumed derived from. The results shown in Table IV indicate that determinants expressed on P815 specific suppressor factor and P815-specific T suppressor cells are not expressed on P815-specific T cytotoxic cells.

Since the preparation of the P815-SF used to induce the anti-P815-SF antisera entailed a final elution step off of a P815 membrane column, there is a possibility that the P815-SF material could also contain small amounts of P815 antigen which was shed off the column along with the bound suppressor factor. It is therefore possible that the antisera possibly contained anti-P815 antigen activity, which could interfere with the interpretation of some of the results reported here. The results shown in Figure 22 and 23 show that the amount of anti-P815 reactivity found in the anti-P815-SF antisera is at most a very small percentage of the total reactivity and probably plays no role in any of the experiments reported here, especially the ones mediated through complement. And of course, since the Balentl-"SF" was prepared in an identical fashion to P815-SF (i.e. final elution off of a P815 membrane column), the antisera prepared to it serves as an excellent internal control for these experiments. It should be restated here that at the concentration used in this work there was no detectable anti-normal DBA/2J activity found in any of the antisera preparations. Also it should be noted that the suppressor factor was prepared from DBA/2J cells primed in vivo, and during

preparation and prior to immunization into the appropriate syngeneic and allogeneic mice, was only suspended in PBS without fetal calf serum. Therefore, it is reasonable to assume that any antibodies being formed in immunized animals would be directed exclusively to antigens of DBA/2J origin. Thus, it seems probable, that the anti-P815-SF antisera from the syngeneic DBA/2J mice is directed to unique determinants (idiotypic?) expressed at or near the antigen binding site of the suppressor factor. The anti-P815-SF antisera from the allogeneic C57BL/6 mice would probably be directed to both receptor site determinants as well as the constant determinants. Similar conclusions were drawn by others using very similar protocols (26, 144-146).

The observation that these antisera do not kill anti-P815 cytotoxic T cells but do kill P815-specific suppressor T cells, implies that these two cell types bear unrelated determinants. As mentioned in the introduction, it is very possible that the specific suppressor factors may be the receptors of specific suppressor T cells. Therefore, since the antisera used contained anti-SF activity (Table II and Figure 18), it is possible that the determinants recognized on the suppressor T cells are associated with the receptor molecules of these cells. The fact that these antisera did not kill anti-P815 cytotoxic T cells implies the possibility that these two P815-specific T cell types may bear unrelated receptor determinants at least at the antigen binding site.

Alternatively, the sensitivity of T cytotoxic cells to the antisera plus complement treatment may reflect a lower density or dif-

ferent arrangement of receptors on their surface when compared to T suppressor cells.

It is becoming very apparent that differential activation of the various T cell subsets can be accomplished by varying the mode of immunization (30,125,147-152). It also seems there may be determinants which selectively react with T helper cells and other determinants which react with T suppressor cells. This can be demonstrated most definitively with small chemically defined molecules in which various fragments of the whole molecule have been shown to selectively activate either T suppressor cells or T helper cells in certain strains of mice (20,153,157). Furthermore, Yamauchi *et al.*, in experiments involving varying immunization protocols and blocking studies, have very recently shown that in one tumor system T suppressor cells and T cytotoxic cells recognize different antigenic determinants (125). In this chapter, these findings are extended by showing that antisera, from both syngeneic DBA/2J and allogeneic C57BL/6 mice, directed against P815-specific suppressor factor reacts with P815-specific T suppressor cells but not specific T cytotoxic cells to this same tumor.

Finally the *in vivo* effect of the anti-P815-SF antisera on P815 tumor growth in DBA/2 mice was assessed. As shown in Figure 20 and Table V only the DBA/2 antisera had any observable effect *in vivo*. This is in contrast to the *in vitro* work which showed that both the syngeneic DBA/2 anti-P815-SF and the allogeneic C57BL/6 anti-P815-SF antisera reacted with suppressor factor/cell determinants. The reason for this is unknown. Although it may be related to the

fact that the allogeneic C57BL/6 anti-P815-SF antisera would likely react with many more determinants present on P815-SF and P815-specific suppressor T cells, than would the syngeneic DBA/2 anti-P815-SF antisera. Therefore, the allogeneic anti-P815-SF antisera may have much more complex activity in vivo than the syngeneic antisera. For example, the allogeneic anti-P815-SF antisera could possibly stimulate P815-specific suppressor T cell induction as well as inhibiting it in vivo. Thus, effectively "cancelling out" its effect in vivo.

CHAPTER V  
SUMMARY DISCUSSION

## CHAPTER V

Summary Discussion

The work reported in the preceeding three chapters involved studies of DBA/2 antigen-specific suppressor T cells and the antigen-specific suppressor factor derived from them. Both the suppressor cells and the suppressor factors specifically inhibit the in vitro generation of DBA/2 cytotoxic T cells for the syngeneic tumor, P815. In Chapter II P815-T<sub>s</sub> and P815-SF were obtained from the thymuses of DBA/2 mice primed previously with P815 tumor cells subcutaneously. The experiments reported in that chapter showed that the P815-T<sub>s</sub> and -SF both expressed Ia<sup>d</sup> determinants and were not H-2 restricted in their ability to effectively suppress the in vitro response to P815 by radiation chimeras of a different H-2 haplotype. The P815-T<sub>s</sub> used in experiments reported in Chapter III were prepared from the spleens of DBA/2 mice injected intraperitoneally with membrane fragments of the P815 tumor cell. Results in that chapter showed among other things that the DBA/2 P815-T<sub>s</sub> expressed the cell surface phenotype of Lyt-1<sup>+</sup>2<sup>-</sup>, whereas the DBA/2 P815-T<sub>c</sub> was Lyt-1<sup>-</sup>2<sup>+</sup>. Chapter IV used P815-T<sub>s</sub> prepared as in Chapter III. P815-SF was then prepared from these suppressor T cells. Syngeneic DBA/2 and allogeneic C57BL/6 antisera was prepared against the P815-SF. Both of the anti-P815-SF antisera reacted in vitro with determinants expressed on the P815-SF and the P815-T<sub>s</sub>, but did not react with the P815-T<sub>c</sub> generated in vitro. Only the DBA/2 anti-P815-SF antisera had any observable effect in vivo on P815 tumor growth in DBA/2 mice.



The studies of some of the genetic properties of the P815-T<sub>s</sub> in Chapter II indicate that this suppressor cell express Ia antigen on its surface. It was also shown that the P815-SF could be absorbed out with anti-Ia<sup>d</sup> antisera, and thus also expressed Ia coded determinants. This last result was also shown in Chapter IV using a different method of preparing P815-SF. There the ELISA assay was used to show that P815-SF prepared from DBA/2 splenocytes expressed H-2<sup>d</sup> and more specifically Ia<sup>d</sup> coded determinants. In every system tested to date the antigen-specific T<sub>s</sub> and the antigen-specific SF produced from it have been shown to express Ia determinants (59-61). Therefore, these results in the DBA/2-P815 system are not surprising and fit in nicely with the assumption that all antigen-specific suppressor T cells and their suppressive factors probably express determinants encoded within the I-J to IE/C region (59-61).

Some of the immunogenetic requirements for the expression of P815-specific suppression were looked at in Chapter II. The results showed that the P815-T<sub>s</sub> and the P815-SF derived from them, were capable of suppressing the in vitro generation of cytotoxicity to P815 by histoincompatible cells. These results showed that the genes within the MHC do not have to be shared by the suppressor cell (or its factor) and its target. In the antigen-specific suppressor systems in which H-2 restriction has been addressed, the published results of others are somewhat equivocal. In most systems involving cell-mediated responses there has been no H-2 restriction found, at least for the first part of the systems activity (see below) (59-61). In one system studied by Moorhead of a antigen-specific SF which

suppresses contact sensitivity to DNP, it was found that the SF required homology at the K and/or D loci of the H-2 with the target cells for activity to be observed (79,80,97). The P815-SF clearly does not need this homology with its target to show suppressive activity.

The work in Chapter III presents evidence of the Lyt phenotype of the cytotoxic T cell and the suppressor T cell in the P815 tumor system in DBA/2 mice. It was found that the primary in vitro generated DBA/2 P815- $T_c$  were  $Lyt-1^{-}2^{+}$ . As was pointed out in the discussion in Chapter III, there may be a difference in the Lyt-1 antigen expression on primary vs. secondary syngeneic tumor- $T_c$  in the DBA/2-P815 system (13,130), and also between syngeneic- $T_c$  and allogeneic- $T_c$  in DBA/2 mice (13). Of course these results probably reflect a purely quantitative not qualitative difference, as all T cells probably express Lyt-1 antigens, although in varying amounts (131).

Even though most antigen specific- $T_s$  in other systems have been shown to express the surface phenotype of  $Lyt-1^{-}2^{+}$  (114-118,120), it is not surprising that the DBA/2 P815- $T_s$  were shown to be  $Lyt-1^{+}2^{-}$ . This is because there have been several reports in a variety of systems of antigen-specific  $T_s$  which are  $Lyt-1^{+}2^{-}$  (42-44, 120, 132,133). As was discussed in Chapter III, these cells are usually found to not be the actual effector cell for suppression but may "induce" other T cells to actively suppress (22,23,44,60,134-136). Thus, the  $Lyt-1^{+}2^{-}$ ,  $Ia^{+}$ , antigen-binding, H-2 unrestricted  $T_{s1}$  inducer is thought to produce a  $Ia^{+}$ , antigen-binding, H-2 unrestricted SF,

which induces a second suppressor T cell,  $T_{s2}$ . This  $T_{s2}$  is  $Lyt-1^{-2+}$ ,  $Ia^{+}$  anti-idiotypic, and H-2 restricted in activity. It produces an  $Ia^{+}$ , anti-idiotypic, H-2 restricted  $SF_2$ , which induces a third suppressor T cell,  $T_{s3}$ .  $T_{s3}$  is  $Lyt-1^{-2+}$ ,  $Ia^{+}$ , idio $type^{+}$ , "final" effector cell, which may suppress in a non-specific fashion, possibly via an interaction with macrophage.

Thus, it can be seen that both the P815- $T_s$  and P815-SF from the thymus or spleen of DBA/2 mice would fit nicely into the above mentioned model of suppression at the  $T_{s1}$  and  $SF_1$  stage. This may at first seem strange since the P815- $T_s$ , which appears after intraperitoneal injection of P815 membrane fragments, does so about 4 days sooner than the P815- $T_s$  induced by P815 cells injected subcutaneously. One explanation for this may be that it takes several days for the antigen load produced by the subcutaneously injected live P815 to equal the amount injected directly via the intraperitoneal injection method. Thus the two P815-specific suppressor T cells may be identical and equivalent to the  $T_{s1}$  in other systems. It may be worth noting that 4-6 days after maximum suppression appears in the thymus, suppression appears in the spleen and lymph nodes of tumor bearing mice (148). This may be an indication of the time it takes for the thymus P815- $T_s$  to "induce" the full suppressor effector function in vivo. The 5 day culture period of the in vitro assay may also allow the "induction" of suppression through the influence of the  $Lyt-1^{+2-}$  P815- $T_s$ .

In the earlier studies of P815- $T_s$  and P815-SF in DBA/2 mice the suppressor cell and suppressor factor were obtained from the thymuses

of DBA/2 mice which had been primed previously with P815 tumor cells (123,124, Chapter II). The suppressive activity of these materials was assayed by their ability to specifically suppress the secondary in vitro cytotoxic response of primed DBA/2 splenocytes to mitomycin-C treated P815 cells. In the work reported in this thesis P815- $T_s$  and P815-SF, either prepared as above from thymocytes or from splenocytes of DBA/2 mice which had been primed previously with P815 tumor membrane extract, could specifically inhibit the primary in vitro cytotoxic response of normal DBA/2 splenocytes to mitomycin-C treated P815. Therefore, the state (primed or virgin) of the cell population on which it acts appears to be unimportant for the P815- $T_s$  or P815-SF activity. Since the number of P815- $T_c$  is probably higher in the P815 primed DBA/2 spleen population than the normal DBA/2 spleen population (130), it would seem that the P815- $T_s$  and P815-SF may act on the helper T cell population. Thus, inhibiting the  $T_h$  provided 2nd signal, which is probably necessary for the P815- $T_c$  to differentiate and become active effectors of cytotoxicity. While this study presents no evidence supporting this possibility, there has been good recent experimental evidence for SF effecting  $T_h$  activity directly (158).

The results presented in Chapter IV show that anti-P815-SF antisera, raised both in syngeneic DBA/2 and allogeneic C57BL/6 mice, could absorb out the P815-SF. These antisera were also capable, in the presence of complement, of eliminating P815- $T_s$  but not P815- $T_c$  from DBA/2 mice. As mentioned in Chapters I and IV, it is likely that the antigen-specific SF may be the receptor molecule of antigen-specific  $T_s$ .

Therefore, since the antisera used contained anti-SF activity, it is very possible that the determinants recognized on the P815-T<sub>s</sub> are associated with the receptor molecules. Thus, the fact that the anti-P815-SF antisera did not kill P815-T<sub>c</sub> implies the possibility that these two P815-specific T cells may bear different receptor determinants, at least at the antigen binding site.

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