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Characterization of Alloantigen Specific Human Suppressor T Cells Generated in Mixed Leukocyte Culture (MLC)

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ABSTRACT

The mixed leukocyte culture (MLC) extended to 11 days provides the best source of allospecific suppressor T cell (Ts) as these cultures no longer autostimulate fresh autologous responder cells and have little cytotoxic activity. The suppressor assay is performed by adding these culture primed Ts to flat-bottom microwell cultures of fresh autologous lymphocytes responding to the same stimulator cells used for Ts induction. These Ts are radioresistant similar to the naturally occurring human Ts of the MLC. The phenotype of the Ts generated in this model is $CD2^+$, $CD8^+$ and 9.3^+. No suppressor activity is found in the lymphocyte subset bearing the CD4 antigen. Studies on the antigen specificity of these alloactivated Ts povide formal evidence that not only Class II but also Class I HLA antigens can suffice as the target antigen(s). Preliminary data is provided to suggest that there are HLA antigens to which a given individual will easily make Ts and HLA antigens to which that individual will not readily produce Ts. It is suggested that this may be due to the genetic background of the responder and thus may be under Ir gene control as in the mouse. The antigen specificity of these Ts has been demonstrated by their failure to suppress the response of autologous lymphocytes to stimulator cells bearing unrelated alloantigens present in the same cultures. However, suppression of the response to unrelated alloantigens can be produced by presentation of those antigen(s) on the same cell bearing the suppressor inducer target antigen.

One of the most important developments in immunology in recent years was the discovery that various immune reactions such as antibody production[40], delayed hypersensitivity[6,74] and rejection of transplanted tumors[29,53] and organs[23,31,46] are regulated by a subset of lymphoid cells called suppressor T cells (Ts). It has been formally demonstrated in animal models that synthetic antigens and naturally occurring bacterial, viral and tumor antigens can induce immunoregulatory suppressor cells. From such studies, it seems to be a general rule that prolonged exposure to antigen, antigen presented in altered form, or antigen given at nonoptimal doses may preferentially induce Ts rather than helper T cells (Th)[29].

A general feature of the Ts system is the involvement of the major histocompatibility complex (MHC) genes in the induction and expression of Ts activity[15,29,72]. This was originally demonstrated with antigens such as the synthetic linear polymer composed of L-glutamic acid, L-alanine, and L-tyrosine (GAT)[40]. Simply stated, mice bearing certain H-2 haplotypes can be shown to be nonresponsive when injected with GAT antigen alone, but most of them do produce anti GAT antibody when the primary immunization is carried out with conjugates of

GAT and methylated bovine serum albumin (MBSA). It was shown that nonresponder mice developed active Ts following immunization with GAT alone, and most excitingly, Ts to GAT were transferrable to naive animals in which they abrogated the immune response to GAT-MBSA.

The genetics of suppression were first evaluated in the mouse using the copolymer of L-glutamic acid and L-tyrosine (GT). With this synthetic antigen, responsiveness in terms of ability to make antibody was confined to a few random-bred mice of the Swiss type, while all of the inbred mouse strains tested were nonresponders. Benaceraff and his colleagues repeated the sequence of experiments with GT that had been performed with GAT[13]. In contrast with GAT, in some of the mouse strains, preimmunization with GT did not alter the recipient's ability to respond to GT-MBSA while in others it did. Analysis of the genetics of occurrence of suppression showed that control of responsiveness is located on an allele within the I region of the H-2 complex. If the mice, who were nonresponders because of suppressor cells were treated with cyclophosphamide two or three days prior to immunization, the animals lost their nonresponsiveness and became responders to the antigen[14].

On the other hand, despite the strong interest in human Ts, knowledge of their genetics and physiology is very much behind such studies made on mice. This is attributable to the difficulties in doing experiments with humans. Whereas immunization of animals can be readily done, this can not be done with human beings. Thus, in humans it is necessary for investigators to avail themselves of the opportunities to study people receiving inoculations, having natural bacterial and viral infections or receiving blood transfusions. Another major obstacle to such studies with human beings is the inability to obtain individuals with identical genetic backgrounds like the inbred mice strains with which to perform genetic analysis. Futher, Ts have been reported to be found in the spleen and lymphnodes of mice but little work has been performed with peripheral blood. Thus, isolation and study of Ts may be difficult in human beings in which investigators are restricted to using peripheral blood almost exclusively as the source of lymphocytes.

Until recent years, human suppressor cell studies were performed using relatively crude assays such as the inhibition of antibody production or blastogenesis by concanavalin A[69] or phytohemagglutinin[21] stimulated cells. Such studies may have some use in the analysis of diseases showing abnormal serum immunoglobulin values such as autoimmune diseases[22] and of lymphocyte function with aging[32]. However, for the study of the *in vivo* immune network, models for the study of immunoregulatory cells with antigen specificity would seem to be much more relevant.

To date, only a few reports have been made on antigen specific Ts in humans. These include the *in vivo* occurrence of Ts specific to antigens of pathogenic organisms such as streptococcus cell wall antigen[51], schistosomal antigen[55] and mycobacterium leprae[48], Ts in lepromatous leprosy patients whose suppression is triggered by lepromin[49], and Ts specific to histocompatibility antigens[3,4,8,17,18,20,47,73]. Studies on such naturally occurring Ts are very significant in that they have demonstrated the presence of antigen-specific Ts in man.

Sasazuki and his colleagues demonstrated an immune suppressor gene to streptococcus cell wall (SCW) antigen in humans[51]. In their studies, low and high responders to SCW antigen were found when lymphoyctes from normal individuals were stimulated with SCW antigen *in vitro*. Family studies demonstrated that the low immune responsiveness to SCW antigen was controlled by an HLA linked inherited dominant gene. In addition, T cells from low responders would suppress the response of HLA DR identical high responder lymphocytes to SCW antigen. And, low responder T cells would respond strongly to SCW antigen after elimination of CD8 positive cells. The investigation of naturally occurring Ts and identification of their target antigens is very difficult and requires examination of many individuals under different conditions with many different antigens.

Sasazuki and Engleman et al[18,47] described a false typing response observed in one-way MLC as being due to an HLA antigen specific Ts. Lymphocytes from a homozygous HLA DW2 multiparous woman failed to respond in MLC to her husband's HLA BW35 homozygous, DW1 typing cells and other HLA Dw mismatched

stimulator cells bearing HLA BW35 or closely related HLA Class I antigens. However, her lymphocytes could respond normally to many other HLA DW1 and other DW disparate stimulator cells lacking the HLA BW35 antigen. In coculture, her T cells suppressed the response of other HLA DW2 homozygous reponders to her husband's lymphocytes in MLC. This same type of Ts was observed by Festenstein's group[20] in their studies of a homozygous typing cell (HTC). Lymphocytes from a healthy individual who was HLA DW2, DW7 failed to respond to HLA DW Fes6 HTCs. But responder cells from an HLA identical sibling of the subject responded well to the HTCs in MLC. In this case, the HTC also bore the HLA BW35 antigen. They showed that the suppressor effects were induced only by HLA BW35 homozygous cells irrespective of their HLA D genotypes. Bean's group[3,4,8] also demonstrated a different type of naturally occurring HLA specific Ts in a patient with bladder cancer. They showed that normal donors whose lymphocyte responsiveness was suppressed by the addition of the patient's cells to their culture all bore HLA B14 on thier surface. The Ts were relatively radioresistant and expressed CD8 antigen on their surface. These three cases showed that naturally occurring MHC specific Ts could be triggered by HLA Class I antigen. If specific Ts could be induced by antigen stimulation *in vitro*, it would greatly facilitate studies of human Ts.

This now seems possible as considerable progress has been made in understanding of the HLA complex and of the MLC reaction. It has been shown that cytotoxic T cells (Tc) are induced in MLC, that their targets are MHC antigens and that they destroy the target cells antigen specifically[2]. With the clarification of the details of the HLA antigen system brought about by a number of workshops and with the establishment of assays of suppressor cell activity resulting from the studies of naturally occurring Ts specific to HLA antigen, many investigators are now beginning to study HLA specific Ts induced during MLC reactions just as previous studies had been made on MLC induced Tc.

In early studies[10,16,25,27,28,36,38,63,66,68], investigators demonstrated that lymphocytes primed in the one-way MLC inhibited the response of freshly prepared lymphocytes in the MLC of the same combination as the original MLC. Engleman's group[10,11,16] has made considerable advances in their study of *in vitro* generated Ts which appear specific for HLA Class II alloantigens. They found that CD4 positive cells activated with allostimulators aquired the ability to stimulate blastogenesis of naive autologous CD8 positive cells. Following this stimulation (in the absence of alloantigen) the CD8 positive cells gained the ability to suppress the response of fresh autologous CD4 positive cells to the original but not to third party stimulator cells in MLC[10]. This body of work represents a rather elegant description of the regulatory networks required for generation of HLA Class II specific Ts *in vitro*. Most of the other reports on allospecific Ts generated in the MLC also deal with HLA Class II target antigens[25,64,66].

This series of reports, while adequately showing the cellular network, fails to account the fact that HLA Class II antigens appear to be the target antigens of Ts induced in their MLC systems whereas HLA Class I antigens are the target antigens of naturally occurring Ts[3,4,8,17,20,47,73]. This is an important difference as HLA Class II antigens are present primarily on certain cells such as monocytes and B lymphocytes, whereas Class I antigens are expressed on almost all cells of the body. Since HLA Class I antigens are expressed on all grafts, the induction of Class I specific Ts would seem more important than that of Class II specific Ts in organ transplantation.

Ts cells may be of great clinical importance. It is known that in organ transplantation, the graft survival rate increases if blood transfusion from the organ donor is given prior to transplantation[64], which strongly suggests the possibility that Ts are being induced. Today, the technique of transfusing donor blood into the recipient before transplantion is frequently used. On the other hand, they may also be important in cancer. The possible explanations of why immunogenic tumors progressively grow in their immunocompetent syngeneic hosts are: 1) they sneak through immunosurveillance[56]; 2) they hide their surface antigens from immune effector mechanisms[70]; 3) they induce the generation of soluble antibody-dependent blocking factors[35]; and 4) they favor the generation of

Ts [50].

North and his colleagues[5,52,53] demomstrated that tumors regressed when intravenous infusions of tumor sensitized T cells from immune mice were given. However, this only occurred when the tumors were growing in T cell deficient recipients and not when they were growing in T cell intact recipients. They found that the tumor induced Ts in T cell intact mice. And, the infusion of immune spleen cells alone into T cell intact mice had no effect on tumor growth. Treatment of the tumor bearing mice with cyclophosphamide alone only caused a temporary halt in tumor progression. In contrast, combination therapy consisting of intravenous injection of cyclophosphamide and tumor immune T cells caused tumors to regress[52]. Green and his colleagues also demonstrated that injections of an anti I-J alloantiserum that recognizes murine Ts caused the reduction of syngeneic tumor growth[30].

Therefore, knowledge of the immunobiology and genetics of Ts in humans may be important not only for transplantation but for devising treatments for cancer patient where Ts may be down-regulating immune responsiveness. Studies on antigen-specific Ts are lagging behind despite the recognition of the importance of Ts in man as described above. We report here on alloantigen specific human Ts which we have induced in MLC and describe their antigen specificity and cell surface antigen profile.

MATERIALS AND METHODS

1. Isolation of peripheral blood lymphocytes (PBL)

Treatment of blood to prevent coagulation is necessary when isolating lymphocytes from the peripheral blood. The methods generally employed use anticoagulants such as heparin or EDTA or removal fibrin by defibrination. The latter method is slightly inferior to the fomer methods in that the yield of lymphocytes is somewhat lower but it is superior in that it does not affect the metabolism of the cells and removes platelets. Therefore, blood preparation by the method of glass bead defibrination was used.

a) Defibrination of blood

Before collecting blood, a 50 ml sterile plastic centrifugation tube was prepared containing about 20 glass beads approximately 5 mm in diameter which had been sterilized in an autoclave. Twenty-five to 35 ml of venous blood was aliquoted to the tube as soon as it was collected, and fibrin and blood platelets were removed by repeated inversion of the tube for 15 min causing adherence of fibrin and platelets to the surface of the glass beads.

b) Isolation of peripheral blood lymphocytes by specific gravity centrifugation

PBL were isolated from healthy volunteers by the method of specific gravity centrifugation. That is, 20 ml of defibrinated blood diluted in an equivalent amount of phosphate buffered saline (PBS) was underlayed with 10 ml of Ficoll-Hypaque (F-H;specific gravity 1.077 ± 0.001) and centrifuged at 400 G for 40 min at room temperature. After centrifugation, the mononuclear cell layer containing the lymphocytes was collected and diluted with Earl's balanced salt solution (EBSS) containing 2.5% fetal calf serum (FCS). The cell suspension was then centrifuged for 10 min at 510 G and the supernatant aspirated. After loosening the cell pellet, EBSS was added and the cells were washed two more times by centrifugation for 10 min at 240 G. The lymphocytes were then suspended in Eagle's MEM (GIBCO) containing 10% heat inactivated fresh normal human male sera, 1% nonessential amino acids (GIBCO), and 1% penstrep (GIBCO) and used for experiments (hereinafter referred to as"test medium").

c) Isolation of peripheral T lymphocytes by nylon wool column

Three parts of defibrinated blood and one part of a 3% gelatin solution in Hanks' balanced salt solution (GIBCO) was mixed well and left to stand upright in a plastic syringe for 45 min at 37°C. The supernatant containing the leukocytes was transferred into a sterile column filled with nylon wool and left to stand in a CO_2 incubator for 30 min. The medium containing non-adhering cells was drained from the column and the nylon was rinsed twice with EBSS. The cells were collected by centrifugation and Tris buffered solution containing 0.83% ammonium chloride was added to the cells at a ratio of 8:1 volume to volume and then incubated for five min at 37°C in order to lyse erythrocytes. After washing three times by centrifugation, the cells were suspended in test medium and used for experiments.

Table 1. Murine Monoclonal Antibodies

Monoclonal Antibody	Function or Antigen Defined	Ig Class	Antigen positive T cells (%)[a]	Molecular weight of antigen	CD Cluster
9E8	Murine Leukemia virus	IgG2a	0	15,000	
9.6	E-receptor	IgG2b	92 ± 3	50,000	CD2
9.3	Helper/inducer plus some CD8$^+$ cells	IgG2a	66 ± 8	44,000	
OKT4	Helper/inducer cells	IgG2b	52 ± 10	62,000	CD4
OKT8	Cytotoxic/suppressor cells	IgG2a	34 ± 9	33,000	CD8
7.2	"Ia" antigen	IgG2b	8 ± 2	29,000 34,000	

a. Mean ± 1SD of measurements made on nylon wool nonadherent peripheral blood cells from 10 healthy adult donors.

2. Monoclonal antibodies

Ever since Köhler and Milstein, utilizing the cell fusion method, succeeded in making a hybridoma which produces antibodies derived from a single clone[41], many monoclonal antibodies reactive with surface antigens of human lymphocytes have been developed. The monoclonal antibodies used in this study are shown collectively in Table 1. Monoclonal antibodies OKT4 (anti CD4) and OKT8 (anti CD8) were developed by Reinherz and Schlossman's group and are marketed by Ortho Diagnostics. OKT4, which reacts with approximately 60% of peripheral T lymphocytes of normal persons, is an antibody which recognizes the helper (or inducer) subset of T lymphocytes[58,60]. OKT8, on the other hand, reacts with about 30% of peripheral T lymphocytes and recognizes a subset of T lymphocytes with cytotoxic or suppressor function[44]. There are few or no peripheral T cells which react to both OKT4 and OKT8 although immature thymocytes may express both antigens[59]. 9E8, 9.6 (anti CD2), 9.3 and 7.2 are monoclonal antibodies reported by Hansen's group[33,34]. These antibodies were made available by the courtesy of Professor John A. Hansen of the University of Washington. 9E8, an antibody which recognizes the P15 antigen of the murine leukemia virus was used as a negative control as this antibody does not react with human cells[54]. 9.6 is an antibody which recognizes receptors for sheep erythrocytes on the surface of human T lymphocytes and reacts with 100% of peripheral T lymphocytes[39]. 9.3, an antibody reacting with approximately 75% of peripheral T lymphocytes from normal persons and reacts with the majority of CD4 positive cells and 30-50% of CD8 positive cells[33]. 7.2, an antibody which recognizes the framework of HLA DR, Class II histocompatibility antigens[34], is reactive with monocytes and B lymphocytes, but its reactivity with peripheral T lymphocytes is normally less than 10%[8].

a) Adjustment of monoclonal antibodies

OKT4 and OKT8 come as lyophilized preparations and contain 0.1% sodium azide (NaN_3) as a preservative which was removed before use. The lyophilized monoclonal antibodies were dissolved in 1 ml of distilled water and diluted 10-fold in PBS containing 10% heat inactivated (HI) FCS. Following dialysis at 4°C for 24 hr against one liter of PBS containing 10% HIFCS, the monoclonal antibodies were sterilized by passing them through a 0.45 μ millipore filter and cryopreserved at −20°C up to the time of use.

9E8, 9.6, 9.3 and 7.2 are produced in ascites obtained by intraperitoneal injection of antibody-producing hybridomas into mice. The ascitic fluid containing antibody was diluted 50-fold in PBS containing 10% HIFCS, sterilized by passage through a 0.45 μ millipore filter and cryopreserved at −20°C.

b) Determination of titer of monoclonal antibodies

A serial dilution of monoclonal antibodies 0.5 ml in volume was prepared in test tubes. 0.5 ml contaning 10 × 10^6 T lymphocytes was added to the tubes and incubated with an equal volume of diluted antibody at 20°C for 45 min. In the case of 7.2, peripheral lymphocytes isolated from F-H were used instead of T lymphocytes. After the incubation with antibody, 1.0 ml of rabbit complement (Pel-Freez Biologicals) which had

been prescreened in advance for low toxicity to lymphocytes was added and the cells were further incubated for 60 min in a 20°C water bath. Then, viable cells were counted by trypan blue staining and cytotoxicity was determined.

Because the cytotoxicity of OKT4 and OKT8 was almost constant up to 320 times dilution and the cytotoxicity of 9.6, 9.3 and 7.2, up to 10,000 times dilution, OKT4 and OKT8 were used at a final dilution of 100 times and 9E8, 9.6, 9.3 and 7.2, at a final dilution of 1,000 times in order to ensure that saturating concentrations of antibody were used.

c) Study of monoclonal antibody positive cells using flow cytometry

Aliquots of one half ml containing 0.5×10^6 lymphocytes were added to small plastic test tubes and the cells were incubated with the diluted antibodies at 0°C for 30 min. The cells were then washed twice by centrifugation and were resuspended in 25 μl of affinity purified FITC conjugated goat anti-mouse IgG (Tago) diluted 20-fold. After washing with PBS containing 1% HIFCS and 0.1% NaN_3, the cells were fixed with a physiological saline solution containing 1% paraformaldehyde and preserved at 4°C in the dark until the analysis was made. Within three days, the surface antigens of the lymphocytes were analyzed using a FACS 440 (Becton Dickinson) flow cytometer with a consort 40 computer.

3. Isolation of lymphocyte subpopulations using monoclonal antibodies

a) Negative selection by monoclonal antibody and rabbit complement treatment

This procedure is the same as the method described in the section 2.b for the determination of titer of monoclonal antibodies but with larger quantities of cells. That is, equivalent volumes of lymphocytes adjusted to 20×10^6/ml and optimally diluted monoclonal antibodies were mixed and incubated at room temperature for 45 min followed by incubation with the complement for 60 min in a 20°C water bath. After centrifugation, two-fold diluted fresh complement was newly added and the cells were incubated for 30 min more. The cells were then resuspended in a Percoll solution (Pharmacia) having a specific gravity of 1.040 in order to remove dead cells and the suspension was. centrifuged for 20 min at 500 G. The viable cells forming a pellet at the bottom of the centrifugation tube were collected and reconstituted to the starting volume with test medium. Hereafter, in this paper, these cells are termed as $CD4^-$, $CD8^-$ and 9.3^- as the monoclonal antibody and complement depleted population.

b) Positive selection by "panning"

An indirect panning technique developed by Wysocki and Sato[75] described in detail elsewhere[8] was used to positively select monoclonal antibody binding lymphocytes. Briefly, anti-mouse IgG (Tago), refined using an affinity column, was diluted in 0.05 M pH 9.5 Tris buffer to a final concentration of 10 μg/ml. Ten ml of this was added to bacteriological petri dish (Fisher) in order to coat the surface. After 40 min at room temperature the dish was rinsed 4 times with PBS and once with a few ml of PBS containing 5% HIFCS and used for panning. Twenty $\times 10^6$ lymphocytes coated with monoclonal antibodies were added to the petri dish and the cells were incubatd for 70 min at 4°C. Then, floating cells were decanted and the cells adhering to the petri dish were collected by flushing with PBS. The purity of the positively selected cells was always greater than 98%. Hereafter, in the paper, these cells are expressed as $CD4^+$ and $CD8^+$.

4. Lymphocyte culture systems

a) Mixed leukocyte culture (MLC)

PBL isolated by F-H specific gravity centrifugation were used untreated as responder cells and as stimulator cells after they were rendered incapable of dividing by irradiation with 2,250 rad using a ^{137}Cesium source irradiator (J.L. Shpard and Associates).

When using flat-bottom microwell plates (Falcon #3040) for determining blast formation, 125×10^3 responder cells and an equal number of stimulator cells were put into each of three wells and cultured in a final volume of 0.2 ml of test medium. When using round-bottom microwell plates (Nunc), 62.5×10^3 responder cells and an equal number of stimulator cells were put into each of three wells and cultured in 0.2 ml of test medium. Culture was performed at 37°C in an atmosphere containing 5% CO_2 saturated with water. After determining the time course of the MLC reaction, cultures were performed for seven days when a flat-bottom plate was used and for six days when a round-bottom plate

was used. Eighteen hr before harvesting the cultures, 0.5 μCi ^{3}H-thymidine (^{3}H-TdR;specific activity 5.0 Ci/m mol, New England Nuclear) was added and lymphocytes were collected using a micro cell harvester (Skatron). The ^{3}H-TdR radioactivity taken up in DNA was measured using a liquid scintillation counter (Packard Tricarb).

b) Induction of cytotoxic lymphocytes

PBL isolated by F-H specific gravity centrifugation were suspended in test medium at a concentration of 1.25 × 10^6/ml. Ten × 10^6 responder cells and an equal number of 2,250 rad irradiated stimulator cells were cocultured in a tisue culture flask (Corning) having a surface area of 25 cm^2 for seven days or eleven days in a CO_2 incubator. Upon completion of culture, the cells were collected and washed twice with centrifugation for 10 min at 200 G. The viable cells were counted with trypan blue and the cells used as cytotoxic effector cells.

c) Assay of cytotoxic lymphocytes

Cytotoxicity was determined according to the method described elsewhere[1]. As target cells, 8—10 × 10^6 peripheral lymphocytes were suspended in test medium to which PHA-P (GIBCO) was added at a final of dilution of 1:800. After culturing for four days, the cells were collected and washed three times with test medium by centrifugation. The cell pellet was suspended in 0.1 ml of HIFCS and 0.1 ml of Na^{51}Cr (2 mCi/ml, specific activity 250-480 Ci/m mol, New England Nuclear) was added and the cells were incubated for 60 min at 37°C. During this incubation, the cells were thoroughly mixed every 15 min to enable uniform ^{51}Cr labelling. The labelling was terminated by adding 10 ml of test medium cooled to 0°C. The cells were then incubated on ice for 30 min more in order for the ^{51}Cr adhering to the surface of the cells without being taken up in the cells to be released. After washing three times with test medium by centrifugation, viable cells were counted with trypan blue and the number of cells adjusted to 100 × 10^3/ml. Then, 0.1 ml of this cell suspension containing 10 × 10^3 cells was dispensed into each well of a round-bottom microwell plate to be used as target cells.

After adding 0.1 ml of effector cells, the plate was centrifuged for one min at 1,000 rpm. The ratios of 50:1, 25:1 and 12.5:1 were normally used as effector cell to target cell ratios (E/T ratios). After culturing in a CO_2 incubator for four hr, the plate was centrifuged again for one min at 1,000 rpm. Then, 0.1 ml of the supernatant of each well was removed and placed into a vial and radioactivity was measured using a gamma counter. For determining spontaneous release, the target cells were incubated with 0.1 ml of the medium instead of effector cells and for determining maximum release, 0.1 ml of test medium containing 0.5% of Triton X-100 was added to the target cells. Cytotoxicity was expressed as:

$$\% \text{ cytotoxicity} = \frac{\text{Test release} - \text{Spontaenous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

When spontaneous release exceeded 20% of maximum release, the results were not used because a high spontaneous release indicates the target cells are not good.

5. Induction of suppressor cells by prolonged MLC

As described in the section 4b on induction of cytotoxic cells, 10 × 10^6 responder cells and 10 × 10^6 irradiated stimulator cells (2,250 rad) were suspended in 16 ml of test medium and cultured in a tissue culture flask with a surface area of 25 cm^2. After culturing for six days, 8 ml of the test medium was replaced with fresh medium. After five more days of culture, the cells were collected. The responder lymphocytes were always prepared from a normal healthy male individual.

a) Measurement of suppressor cell activity using DNA synthesis as an index

Indicator MLCs were set up in the microtest plate in the same manner as when conducting a routine MLC reaction. That is, 125 × 10^3 responder cells and an equal number of stimulator cells were put in each well when the flat-bottom microwell plates were used and 62.5 × 10^3 of each of the two cells when the round-bottom microwell plates were used. To these were added 11 day alloactivated cells prepared as described above which were irradiated to 2,250 rad and used as modulator cells. In some cases, EB virus transformed B cell lines were used as stimulator cells. Such MLCs were performed with 125 × 10^3 responder cells and 31

$\times$ 10^3 6,000 rad irradiated B cells in a flat-bottom plate. The EB virus transformed B cell lines used in this study were T5-1 and 6.6.5. that were generously provided by Dr. Paul Gladstone and Dr. Don Pious (University of Washington). The B cell line 6.6.5. is an HLA A2 loss mutant cell line derived from low dose irradiated T5-1[42]. After culturing for seven days, ^{3}H-TdR radioactivity was measured as is normally done in MLC. Suppression is expressed as:

$$\% \text{ suppression} = \left(1-\frac{\text{MLC in the presence of alloactivated cells}}{\text{MLC in the absence of allocativated cells}}\right) \times 100$$

b) Measurement of suppressor cell activity using cytotoxicity as an index

Ten $\times$ 10^6 responder cells and 10 $\times$ 10^6 stimulator cells were cultured in a tissue culture flask, and, to this, 10 $\times$ 10^6 11 day alloactivated cells irradiated to 2,250 rad were added. The cells were cultured for seven days in 16 ml of test medium, and, as described in the section on cytotoxicity, a cytotoxicity test was performed using target cells which were ^{51}Cr labelled PHA blasts of lymphocytes used as stimulator cells.

Table 2. HLA Phenotypes of the Normal Blood Donors

Donor	HLA A	HLA B	HLA DR
A	11,26	35,40	4, 6
B	26,31	5,40	4, 8
C	2, 3	7,12	2, 4
D	2, -	7,12	2, 3
E	1, 2	5,17	6, 7
F	24,29	7,12	7, -
G	2, -	7,12	3, 6
H	1, -	8, -	3, 4
I	2,24	7,22	4, 7
J	29,30	12,18	3, 4
K	3,24	5,35	2, -
L	3, -	7,40	2, 4
M	2, -	17,35	4, 7
N	24,25	7,27	1, -
O	3,24	5,12	4, 7
P	1,11	15,35	-, -
Q	24, -	35, -	4, 6
R	1, 3	8,37	3,10

6. HLA typing of lymphocyte donors

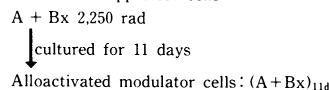
HLA A, B and DR typing was kindly performed by Dr. John A. Hansen (University of Washington and Histocompatibility Laboratory, Fred Hutchinson Cancer Research Center, Seattle Washington). The HLA typing of the normal blood donors used in this study are shown in Table 2. For simplicity of presentation, each individuals's PBL were assigned a letter designation to identify them.

RESULTS

The protocol adapted from Rode and Gordon[63] for our study of the specificity and genetics of human alloantigen reactive suppressor cells is shown in Fig. 1. To induce alloantigen reactive suppressor cells, PBL from an individual designated by alphabetical letters such as"A" and irradiated PBL from an HLA unrelated individual designated"B" are cultured in a flask for 11 days, after which the lymphocytes are collected, irradiated and used in assays as alloactivated modulator cells (hereinafter abbreviated as (A+Bx)11dx). The method employed for detection of suppressor activity was to perform a fresh MLC of A-derived lymphocytes and irradiated B-derived lymphocytes as a baseline MLC and the suppressor activity was assessed by the MLC inhibition produced by the addition of (A+Bx)11dx as third party modulator cells in the MLC. The alloactivated modulator cells were irradiated to prevent their proliferation. In the assay, responder cells were usually PBL from the same individual used as the responder cells for induction of alloactivated cells. Table 3 shows the assay results when performed in this manner.

EXPERIMENTAL PROTOCOL

A. Generation of suppressor cells:



B. Assay of suppressor cells:

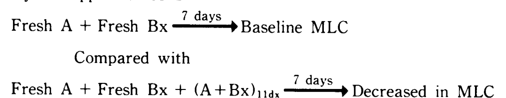


Fig. 1.

Table 3. Specific Suppression of the MLC by Irradiated Alloactivated Cells[a]

Responder	Stimulator	Alloactivated Modulator	^{3}H-TdR MLC Response[b]	% Suppression
A	Bx	–	36,562 ± 2,945	
A	Bx	(A+Bx)11dx	8,916 ± 6,271	76
A	Bx	(A+Cx)11dx	36,557 ± 2,428	0
A	Cx	–	33,162 ± 731	
A	Cx	(A+Bx)11dx	35,937 ± 911	–8
A	Cx	(A+Cx)11dx	8,061 ± 2,198	76
A	–	–	120 ± 1	
A	–	(A+Bx)11dx	793 ± 192	
A	–	(A+Cx)11dx	311 ± 177	

a. Lymphocytes from individual A were primed *in vitro* against irradiated (2,250rad) stimulator cells from individuals Bx or Cx. Alloactivated cells were collected on day 11, irradiated (2,250rad) and tested for their suppressive activity on the MLC response of 125 × 10^3 fresh autologous responder cells and 125 × 10^3 irradiated (2,250rad) original or unrelated stimulator cells in triplicate in flat-bottom microwells for 7 days in the presence of 125 × 10^3 modulator cells.

b. Mean cpm ± 1 SD.

Inhibition of the MLC by alloactivated cells is specific for the original stimulator lymphocytes. In this example, two sets of bulk MLCs were prepared for induction of allostimulated cells. The 11 day bulk cultures were performed using A responder lymphocytes and irradiated HLA unrelated stimulator cells from donor B and C for 11 days. As shown in Table 3, the fresh indicator MCL of A lymphocytes responding to B stimulator cells was 36,562 cpm when no modulator cells were present in the assay. When irradiated alloactivated modulator cells (A+Bx)11dx were added to the MLC of A responding to Bx, they strongly suppressed the response (i.e., 8,916 cpm, 76% suppression). But, when the control alloactivated cells (A+Cx)11dx were added to the MLC of A responding to Bx stimulators there was no inhibition of the response (i.e., 36,557 cpm, 0% suppression). The MLC between A responders and Cx stimulators showed a response of 33,162 cpm in the absence of modulator cells and was inhibited by the addition of specific alloactivated modulators (A+Cx)11x (76% suppression), but not by control modulators (A+Bx)11dx (–8% suppression). It should be stressed that suppression was not observed when the alloactivated cells were added to fresh autologous responders in the indicator MLC when HLA unrelated stimulator cells were used. That is, alloactivated cells derived from bulk MLC suppressed the MLC response only to the original priming allostimulators or to cells sharing alloantigens (see below). Responder cells from A showed no response to either alloactivated cells (A+Bx)11dx or (A+Cx)11dx when cultured in the absence of stimulators, demonstrating the absence of autostimulation by 11 day primed cells.

In order to optimize the assay to give the good sensitivity and specificity shown in Table 3, many aspects of the system had to be studied. The effect that the shape of microtest plate wells was studied first as different investigators have used different microtest wells. The round-bottom microwell plate has the advantage of needing a smaller number of cells and is often used in clinical MLC tests. However, its disadvantage is that there can be an excessive concentration of cells in the bottom of the well which might give rise to artifactual results due to crowding. Accordingly, the results of tests using flat-bottom microwell and round-bottom microwell plates were compared. With flat-bottom microwells, 125 × 10^3 responder cells and stimulator cells were cocultured in each well and, with the round-bottom microwells, 62 × 10^3 responders and stimulators were cultured in each well (Fig. 2). With the use of the flat-bottom microwell plate, the suppression of the MLC to the priming stimulators was somewhat weaker than that seen in the round-bottom microwell plate (59% vs 90% at a responder to

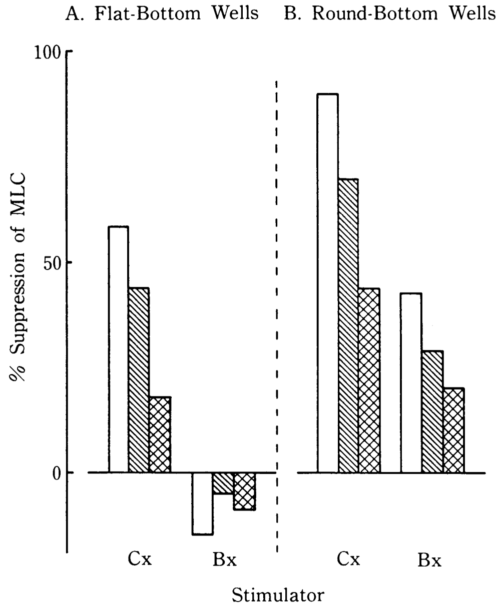


Fig. 2. PBL from individual A were primed *in vitro* against irradiated stimulator cells from individual C. The alloactivated cells were cellected on day 11, irradiated and tested for their suppressive activity in MLC in which 125 × 10^3 fresh A responders and 125 × 10^3 fresh Cx stimulators or 62 × 10^3 responders and 62 × 10^3 stimulators were incubated in flat-bottom microwells (A) for 7 days or in round-bottom microwells (B) for 6 days respectively. The responder to stimulator to modulator ratio was 1:1:1 (□), 1:1:1/2 (▨) and 1:1:1/4 (▩).

modulator ratios of 1:1), but no suppression was seen in the MLC against unrelated stimulators. That is, perfect specificity was observed with use of the flat-bottom microwell plate in this three-cell suppressor assay. In contrast, using the round-bottom microwell plate, significant suppression was observed in the MLC not only to the original stimulators, but also to the HLA unrelated simulators (43%, 29% and 20% at responder to modulator ratios of 1:1, 1:1/2 and 1:1/4 respectively). Thus the flat-bottom microwell plates were used for the study of alloactivated Ts reported here.

When alloactivated modulator cells are not irradiated, they can be activated again by the stimulator cells and undergo blastogenesis and incorporate ^{3}H-TdR in the three-cell assay (data not shown). When more than two kinds of cells are allowed to react in the same well, interpretation of the test results becomes impossible. For these reasons alloactivated cells are usually irradiated before use in such tests. Alloactivated cells irradiated to 4,500 rad, 2,250 rad, 1,125 rad, 562 rad, 281 rad and 0 rad were added to the MLC with specific and HLA unrelated control stimulator combinations as shown in Fig. 3. The suppression of MLC to specific stimulators was relatively resistant to irradiation and showed 50% suppression even with irradiation of 4,500 rad. In contrast, when the alloactivated cells were not irradiated there was a marked suppression of the MLC response to both the specific and the unrelated stimulators. But, after irradiation of 1,125 rad or more, the suppression of the MLC to the unrelated stimulators disappeared and specific suppression still remained when alloactivated cells were irradiated over 1,125 rad. Thus we chose to use 2,250 rad for our experiments.

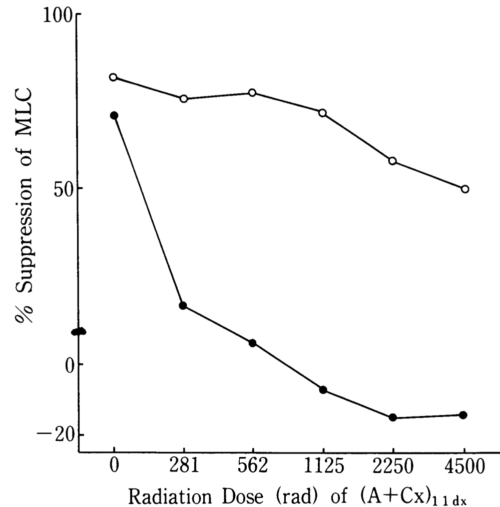


Fig. 3. PBL from individual A were primed *in vitro* against irradiated stimulator cells from individual C. The alloactivated cells were cellected on day 11, irradiated at doses from 0 rad to 4,500 rad and tested for their suppressive activity in MLC in which fresh autologous responder cells and irradiated fresh original stimulator cells (Cx) (○—○) or unrelated stimulator cells (Bx) (●—●) were incubated for 7 days. The responder to stimulator to modulator ratio was 1:1:1.

The responder to stimulator to modulator ratio in the above experiments was 1:1:1. The dose response of suppression produced by alloactivat-

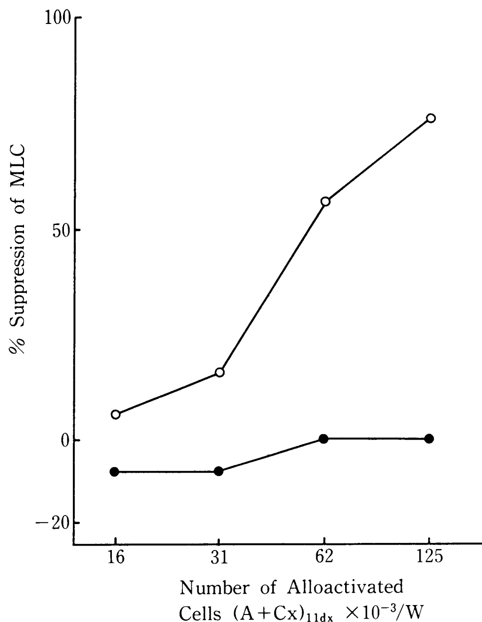


Fig. 4. PBL from individual A were primed *in vitro* against irradiated stimulator cells from individual C. The alloactivated cells were cellected on day 11, irradiated and titrated for suppressive activity in MLC consisting of 125 × 10^3 fresh autologous responder cells and 125 × 10^3 fresh irradiated (2,250 rad) original stimulators (Cx) (○——○) or unrelated stimulators (Bx) (●——●). The MLCs of A + Cx and A + Bx were 33,162 cpm and 36,562 cpm respectively.

ed cells was studied. Specific suppression attenuated as the number of modulator cells decreased (Fig. 4), showing that specific suppression is dose-dependent.

Experiments were undertaken to determined the variability among individuals for the ability to make allosensitized cells that produce inhibitory effects on the responsiveness of fresh autologous to the priming alloantigen. Allostimulated cells recovered from 11 day MLCs performed with PBL from three different individuals sensitized with the same irradiated stimulator cells were washed, irradiated to 2,250 rad and tested for inhibitory effects on the responsiveness of fresh autologous cells. Lymphocytes from the three individuals A, B and G were primed *in vitro* against stimulator cells from Px (Table 4). Alloactivated modulator cells, (A+Px)11d, (B+Px)11d and (G+Px)11d were collected on day 11, irradiated 2,250 rad and added to fresh responder cells in the suppressor assay. That is, fresh A, B and G lymphocytes were stimulated by irradiated Px lymphocytes and autologous modulator cells were added at a ratio of 1:1 modulator to responder. As can be seen in Table 4, the MLC response of A to Px was 18,050 cpm. The addition of the alloactivated modulator (A+Px)11dx to the MLC decreased the response to 2,391 cpm producing a 87% inhibition of the responsiveness. Similar results were obtained by addition of alloactivated cells (B+Px)11dx to fresh B responding to Px(95% inhibition) and addition of (G+Px)11dx to G responding to Px(78% inhibition). This experiment shows that PBL from different donors can be induced to make alloprimed cells with suppressive activity.

Table 4. Effect of Addition of Alloactivated Modulators on the MLC Response[a]

Responder	Stimulator	Alloactivated Modulator	^{3}H-TdR MLC Response[b]	% Suppression
A	Px	–	18,050 ± 3,243	
A	Px	(A+Px)11dx	2,391 ± 586	87
B	Px	–	22,189 ± 2,787	
B	Px	(B+Px)11dx	1,070 ± 359	95
G	Px	–	15,507 ± 1,099	
G	Px	(G+Px)11dx	3,425 ± 583	78

a. Lymphocytes from individuals A, B and G were primed *in vitro* against irradiated stimulator cells from individual Px. Alloactivated cells were collected on day 11, irradiated and tested for their suppressive activity in the MLC as described in Table 3.

b. Mean cpm ± 1 SD.

Table 5. Effect of Addition of Alloactivated Modulators on the MLC Response[a]

Responder	Stimulator	Alloactivated Modulator	^{3}H-TdR MLC Response[b]	% Suppression
A	Bx	–	78,114 ± 3,645	
A	Bx	(A+Bx)11dx	53,677 ± 2,664	31
A	Cx	–	57,374 ± 9,864	
A	Cx	(A+Cx)11dx	19,730 ± 3,867	66
A	Ix	–	58,619 ± 3,224	
A	Ix	(A+Ix)11dx	26,665 ± 5,629	55
A	Hx	–	48,503 ± 4,361	
A	Hx	(A+Hx)11dx	10,703 ± 4,128	78

a. Lymphocytes from individual A were primed *in vitro* against irradiated stimulator cells from individuals Bx, Cx, Ix or Hx. Alloactivated cells were collected on day 11, irradiated 2,250 rad and tested for their suppressive activity in the MLC as described in Table 3.

b. Mean cpm ± 1 SD.

Next, experiments were carried out with responder cells from a single individual A which were primed with stimulator cells from 4 different healthy donors in MLC (Table 5) in order to determine if a given individual could make suppressor cells to more than one stimulator cell. In this case also alloactivated modulators (A+Bx)11dx, (A+Cx)11dx, (A+Ix)11dx and (A+Hx)11dx all significantly inhibited the responsiveness of fresh A PBL to the appropriate stimulator cell in MLC. However, some alloactivated modulator cells were more potent suppressors of the MLCs than were others, eg., (A+Hx)11dx produced 78% suppression versus (A+Bx)11dx which produced 31% suppression.

Preliminary experiments have been made concerning the genetic variation in the ability to make Ts. PBL from A or Q were stimulated with the same irradiated Dx stimulator cells for 11 days, harvested and tested their ability to suppress the MLC of fresh autologous responder cells to different stimulators that shared various HLA antigens with the original stimulator cells. As can be seen in Fig. 5, (A+Dx)11dx suppressed the response to stimulator cells having HLA A2, HLA B7 and HLA DR2. In contrast, alloactivated cells (Q+Dx)11dx, which were induced with the same original stimulators suppressed the response only to the original stimulator and stimulators sharing HLA DR2, and no suppression to HLA A2 or HLA B7 was seen. The HLA type of responder Q was A24, B35, DR4 and DR6 and was totally unrelated to the HLA A2 and B7 on the stimulator cells.

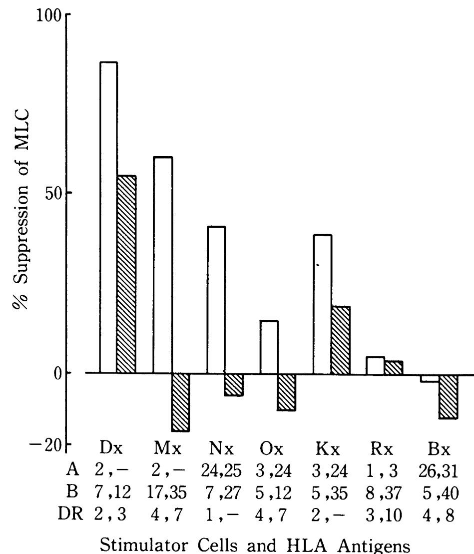
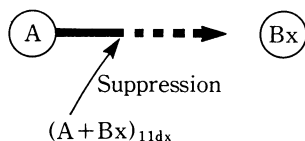


Fig. 5. PBL from individual A (HLA A11,26 B35,40 DR4,6) or Q (HLA A24,- B35-, DR4,6) were primed *in vitro* against irradiated stimulator cells from individual Dx. The alloactivated cells were collected on day 11, irradiated and tested for their suppressor activity in MLCs in which fresh responder cells from A (□) or Q (▧) were stimulated with original stimulator cells (Dx), unrelated stimulator cells (Bx) or stimulator cells (Mx, Nx, Ox, Kx and Rx) that shared only one HLA antigen with the original priming stimulator cells. The responder to stimulator to modulator ratio was 1:1:1.

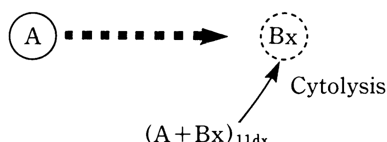
Various possible mechanisms could explain this specific inhibition of the MLC response (Fig. 6).

Possible Explanation for a Reduced MLC in the Presence of Alloactivated Cells Are :

1. Suppressor cells in alloactivated cells



2. Cytotoxic cells in alloactivated cells



3. Change in MLC Kinetics

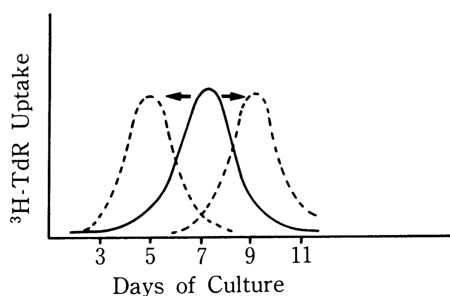


Fig. 6.

The first possibility is that in the alloactivated population there are suppressor cells which specifically inhibit the responder cells from reacting to the stimulator cells. Secondly, there is the possibility that specific destruction and reduction of the number of stimulator cells by cytotoxic cells, which are known to be induced in MLC, might result in the inhibition of the MLC. The third possibility is that the kinetics of the MLC might change due to the addition of alloactivated cells leading to an apparent decline of the MLC blastogenesis when measured on the seventh day.

First, the possibility of the inhibition was due to cytotoxicity of the alloactivated modulator cells for the stimulator cells was investigated. It has been demonstrated by many investigators that the peak of cytotoxic cells induced in MLC occurs on the sixth or seventh day of culture and little cytotoxic activity is remaining by the 11th day. However, there is the possibility that

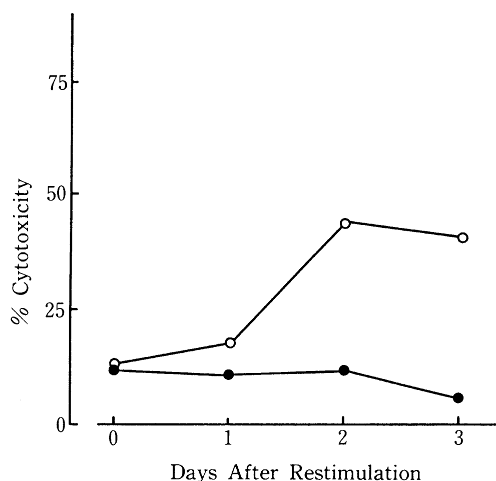


Fig. 7. Responder lymphocytes were primed *in vitro* against irradiated stimulator cells for 11 days. The alloactivated cells were collected, not irradiated (○——○) or irradiated to 2,250 rad (●——●), restimulated by specific stimulators and tested for their cytotoxicity for specific targets on day 0,1,2 and 3 after restimulation. E/T ratio was 50:1.

the 11 day alloactivated cells which are very much attenuated in their cytotoxicity might be restimulated by the specific stimulators and again develop significant cytotoxicity. Therefore, to study this, 11 day alloactivated cells were reexposed to stimulator cells at a ratio of 1:1 (at the same ratio as used in the suppressor assay), and their cytotoxicity for target PHA blasts derived from the stimulator PBL were tested at 0, one, two and three days after culture (Fig. 7). Although the 11 day alloactivated cells showed little or no cytotoxicity before reculture, non-irradiated alloactivated cells developed strong cytotoxicity (44%) by the second day of culture and were still highly cytotoxic on the third day. On the other hand, no significant cytotoxicity was generated by restimulation of irradiated 11 day alloactivated cells which exhibit suppressive activity in MLC.

Also, if the inhibition of the MLC was due to a cytotoxic effect of the alloactivated modulator cells on the stimulator cells, it might be reversed by increasing the number of stimulating cells. When 125 $\times$ 10^3 responder cells were cultured with 31, 62, 125, 250 and 500 $\times$ 10^3 stimulator cells there was a dose dependent increase in the indicator MLC. However, when alloactivated modulator cells were added, the magnitude

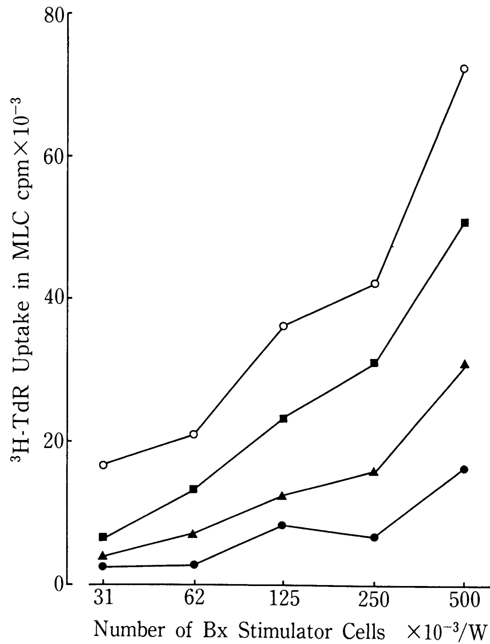


Fig. 8. The effect of stimulator cell concentration on suppressor activity was assessed with 125 × 10^3 responder cells and 31 × 10^3 to 500 × 10^3 irradiated original stimulator cells in the absence (○—○) or presence of 31 × 10^3 (■—■), 62 × 10^3 (▲—▲) or 125 × 10^3 (●—●) alloactivated modulator cells.

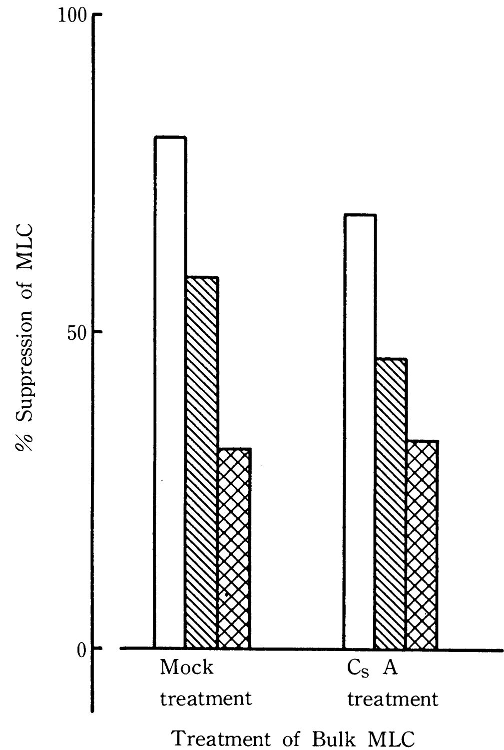


Fig. 9. Responder lymphocytes were primed *in vitro* against irradiated stimulator cells in the absence or presence of 1 μg/ml cyclosporine A. The alloactivated cells were collected on day 11, irradiated and tested for their suppressive activity in MLC in which fresh autologous responder cells and irradiated original stimulator cells were incubated for 7 days. The responder to stimulator to modulator ratios were 1:1:1 (□), 1:1:1/2 (▨) and 1:1:1/4 (▩). The MLC response in the absence of modulator cells was 20,425 cpm.

of the MLC suppression was relatively constant within the range tested despite the increase in stimulator cell dose (Fig. 8).

Cyclosporine A (CsA) is known to inhibit induction of Tc both *in vivo* and *in vitro* and has been reported to have no effect on the induction of Ts[7]. Thus, as another approach the alloactivated cells were induced in MLC in the presence and absence of 1 μg/ml CsA, a concentration at which cytotoxic cells are inhibited and blastogenesis is significantly reduced in MLC (data not shown). As can be seen in Fig. 9, When CsA was not added, there was 81% suppression. There was 69% suppression when the alloactivated cells had been cultured with CsA which demonstrated that there is little effect on suppression due to addition of CsA. However, the cytotoxicity of the alloprimed cells was completely eliminated (data not shown). Data derived from the above three experimental approaches make it evident that inhibition of MLC by alloactivated cells is not due to damage of the specific stimulator cells by cytotoxic cells as had been originally proposed by Fitch, et al[24].

Next, experiments were performed to determine whether the kinetics of MLC were affected by the addition of alloactivated cells (Fig. 10). The MLCs of A+Bx and of A+Dx showed peaks of blastogenesis on the seventh day of culture. When alloactivated cells (A+Bx)11dx were added, the blastogenic response to the original stimulator Bx was eliminated at all time periods, and there was no change in kinetics or shift in the peak of reaction. The kinetics and strength of the MLC to the HLA unrelated stimulator Dx did not change with the addition

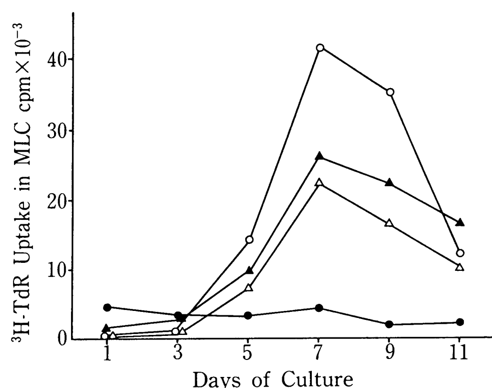


Fig. 10. The kinetics of MLC response in the presence of alloactivated cells. Fresh A responders and original stimulators (Bx) were incubated in the absence (○——○) or in the presence (●——●) of irradiated alloactivated cells (A + Bx)11dx. Fresh A responders and unrelated stimulator (Dx) were incubated in the absence (△——△) or in the presence (▲——▲) of irradiated alloactivated cells (A + Bx)11dx. Triplicate cultures were harvested after varying periods of incubation.

Table 6. Change of Cell Phenotype Over the Course of MLC

Monoclonal Antibody	% Positive Cells[a] Days After Stimulation in MLC 0	4	7	11
CD2⁺	79	84	98	99
9.3 ⁺	51	77	84	72
CD4⁺	31	52	71	67
CD8⁺	21	22	17	13
7.2 ⁺	17	9	41	64

a. Monoclonal antibody positive cells were defind by flow cytometry on a FACS IV.

of alloactivated (A+Bx)11dx, and the peak of the MLC reaction was observed on the seventh day, the same as in the baseline MLC.

T lymphocytes are known to proliferate in the MLC. The phenotype of lymphocytes during the course of the MLC reaction are shown in Table 6. CD2⁺ cells (T cells) are practically the only cells present on the seventh day of MLC reaction. CD4⁺ cells (helper/inducer cells) increased up to the seventh day (71%) and then decreased somewhat (67%) by the 11th day. CD8⁺ cells (cytotoxic/suppressor cells) decreased over time to 13% on the 11th day. 9.3⁺ cells like CD4⁺ cells, increased up to the seventh day and decreased a little by the 11th day. 7.2 is an antibody that recognizes the framework antigen of HLA DR and is present on B cells and monocytes and on less than 10% of resting peripheral T cells. 7.2⁺ cells increased to 64% positive upon 11 days stimulation showing that the majority of T cells were activated.

The FACS patterns of cells obtained from 11 day MLC are shown in Fig. 11. CD2⁺ cells were distributed with two peaks, and bright fluorescent cells were observed to be relatively common. Intensely fluorescent cells accounted for the great majority of CD8⁺ cells and weakly fluorescent cells accounted for great majority of CD4⁺ cells. 7.2⁺ cells were widely distributed in their fluorescence intensity from weak to strong.

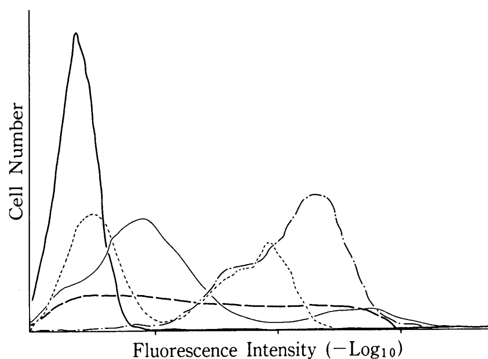


Fig. 11. Immunofluorescence profile of alloactivated cells (A + Ex)11d stained with monoclonal antibodies 9E8 (—), anti CD2 (—·—), anti CD4 (----), anti CD8 (—) and 7.2 (——).

Eleven day alloactivated cells were treated with monoclonal antibodies and complement in order to eliminate selected populations or subsets. As shown in Fig. 12, with mock or complement treatment alone, suppression of 73% and 65% respectively was observed. Strong suppressor activity was also evident after treatment with anti CD4 and complement (60%). However, when the alloactivated suppressor cells were treated with anti CD8 and complement, the MLC reaction was restored and the suppressor activity was markedly decreased with suppression of only 18% at a responder to modulator ratio of 1:1. This data suggest that suppression is dependent on CD8⁺ cells.

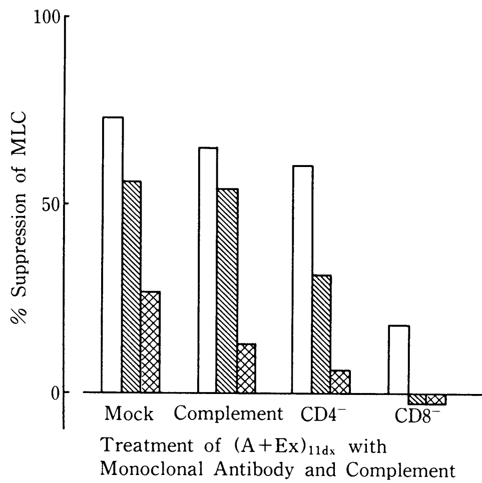


Fig. 12. The alloactivated cells were collected on day 11, treated with monoclonal antibodies and complement and irradiated before assessment of their suppressive activity on the proliferative response in MLC consisting of fresh autologous responder cells and the original stimulator cells. The alloactivated cells treated with monoclonal antibody and complement were reconstituted to the original starting volume as described in Materials and Methods. Suppression was not observed when alloactivated cells were added to the MLC with unrelated stimulator cells (data not shown). The responder to stimulator to modulator ratios were 1:1:1 (□), 1:1:1/2 (▧) and 1:1:1/4 (▩).

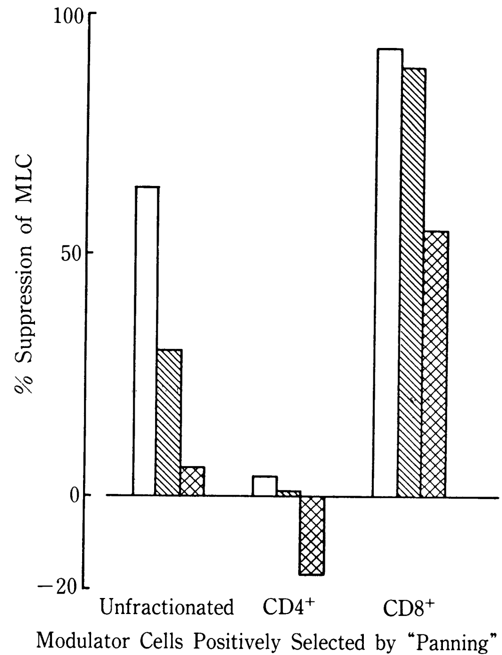


Fig. 13. The alloactivated cells were collected on day 11, used unfractionated or positively selected (panning) by anti CD4 or anti CD8 and irradiated before assessment of their suppressive activity on the proliferative response in MLC to the original stimulator cells. Suppression was not observed when alloactivated cells were added to the MLC with unrelated stimulator cells (data not shown). The responder to stimulator to modulator ratios were 1:1:1 (□), 1:1:1/2 (▧) and 1:1:1/4 (▩).

In order to directly demonstrate the phenotype of the T cell population with the suppessor activity, suppressor activity was determined after selective enrichment of $CD4^+$ or $CD8^+$ cells by panning (Fig. 13). Unfractionated alloactivated cells induced by 11 day MLC showed strong suppressor activity. $CD4^+$ cells panned from the alloactivated cells had no suppressor activity at all. In contrast, positively selected $CD8^+$ cells showed stronger suppressor activity than the unfractionated cells. No suppression was shown by either the $CD4^+$ or the $CD8^+$ cells in the MLC where HLA unrelated stimulator cells were used (data not shown).

Owing to the poor recovery of 9.3^+ cells by the panning method, 9.3^+ and 9.3^- populations were obtained by cell sorting using a FACS IV flow cytometer. As shwon in Fig. 14, alloactivated cells treated with 9.3 and goat anti-mouse IgG and not separated showed 23% specific suppression. 9.3^+ cells that had been sorted had increased suppressor activity (47%). On the other hand, 9.3^- sorted cells showed no suppression at all. When the MLC was performed with HLA unrelated stimulator cells, no suppression was produced by the addition of alloactivated cells treated either with 9.3 and goat anti-mouse IgG or the 9.3^+ or 9.3^- sorted cells as modulator cells.

Specificity of alloactivated Ts cells

Experiments were performed in order to determine the target antigens of the alloactivated suppressor cells. The suppressive effects of alloactivated cells on fresh autologous PBL responding to different stimulator cells are shown in Table 7. (A+Bx)11dx, which inhibited the MLC of A to the original stimulators Bx, showed no suppression of A's response to Cx, Dx, Gx, Hx and Ix which are stimulator cells

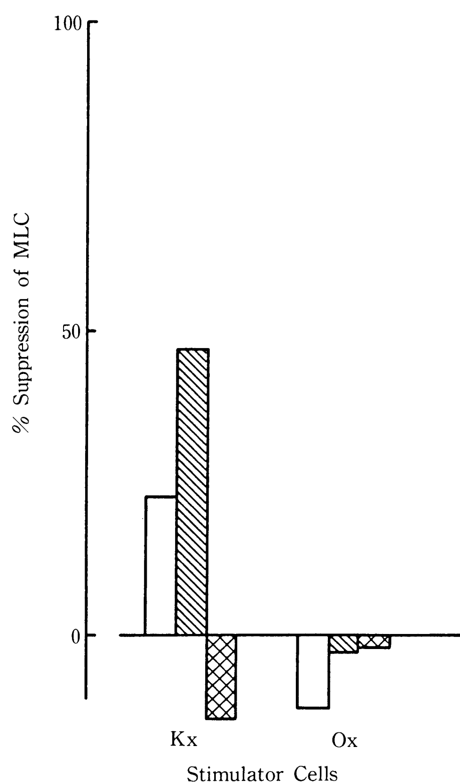


Fig. 14. The alloactivated cells were collected on day 11 and treated with 9.3 and Goat anti-mouse IgG, then the cells were used unfractionated (▭) or separated by sorting on a FACS IV into 9.3⁺ (▧), or 9.3⁻ (▩) and irradiated before assessment of their suppressive activity on the proliferative response in MLC to the original stimulator cells (Kx) or unrelated stimulator cells (Ox). The responder to stimulator to modulator ratio was 1:1:1.

that do not share any known HLA antigens with the original priming stimulators. On the other hand (A+Cx)11dx not only suppressed the response to the original stimulators, but suppressed the response of A to other stimulator cells sharing HLA A2 and B7 with it. In this case also, there was no suppression of the response to Bx and Hx which are stimulators that do not share any known HLA antigens with the priming stimulators. (A+Ex)11dx suppressed the response of A to stimulators sharing HLA A2 and B5 with the original stimulator. However, no suppression was evident when Fx sharing HLA DR7 or Hx sharing HLA A1 was used as a stimulator. Thus, suppression was observed in some but not all cases when HLA antigens were shared by the stimulators.

In order to more clearly define the antigen specificity, alloactivated suppressor cells were made with Dx (HLA A2, B7, B12, DR2, DR3) as the original stimulator and A as the responder and tested against stimulators which shared one known HLA antigen each (Fig. 15). Suppression was observed not only to the original stimulator but also to Mx which shared HLA A2, Nx which shared HLA B7, and Kx which shared HLA DR2. However, hardly any suppression was observed against Ox which shared HLA B12 and Rx which shared HLA DR3. Suppression was not observed to Bx which shared no HLA antigens. The HLA type of responder A is A11, A26, B35, B40, DR4 and DR6 and thus completely different from the priming stimulator cells from Dx.

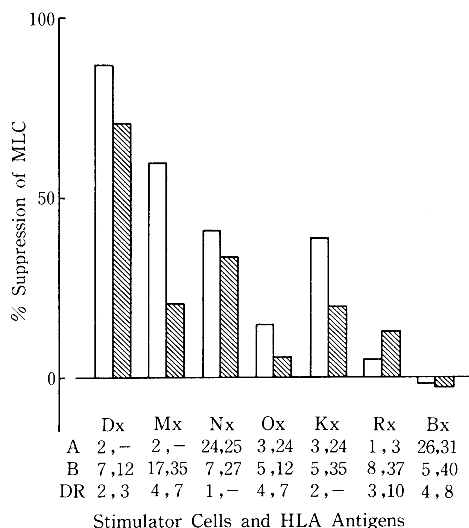


Fig. 15. PBL from individual A(HLA A11,26 B35,40 DR4,6) were primed *in vitro* against irradiated stimulator cells from individual Dx(HLA A2-B7,12 DR2,3). The alloactivated cells were collected on day 11 irradiated and tested for their suppressive activity in MLCs in which fresh autologous responder cells (A) were stimulated with irradiated original stimulator cells (Dx), unrelated stimulators (Bx) or with stimulator cells (Mx, Nx, Ox, Kx and Rx) that shared only one HLA antigen with the original priming stimulator cells. The responder to stimulator to modulator ratios were 1:1 (▭) and 1:1/2 (▧).

Table 7. Target HLA Antigen of Alloactivated Ts

Responder	Alloactivated Modulator[a]	Stimulator	Target HLA Antigen shared with original stimulator			Degree of Suppression[b]
A	(A+Bx)11dx	Bx	A31	B5	DR8	3+
		Cx				–
		Dx				–
		Gx				–
		Hx				–
		I x				–
A	(A+Cx)11dx	Cx	A2,3	B7,12	DR2	3+
		Bx				–
		Gx	A2	B7,12		2+
		Hx				–
		I x	A2	B7		1+
A	(A+Ex)11dx	Ex	A1,2	B5,17	DR7	2+
		Dx	A2			2+
		Fx			DR7	–
		Hx	A1			–
		J x				1+
		Kx		B5		1+
		Lx				–
		Mx	A2	B17	DR7	2+
A	(A+Lx)11dx	Lx	A3	B7	DR2	2+
		Ex				–
		Hx				–
		Mx				–

a. Responder to stimulator to modulator ratio was 1:1:1
b. Symbols indicate the suppression is <10%(–), 10–40%(1+) 41–70%(2+), or >70%(3+).

Alloactivated cells (A+Cx)11dx had shown good suppression that appeared to be against HLA A2. Accordingly, an experiment was made using informative cell lines to ascertain whether HLA A2 was a target antigen (Fig. 16). T5-1 is an EB virus transformed B-cell line and its HLA type is A1, A2, B8, B27, DR1 and DR3. 6.6.5 is an HLA loss mutant cell line derived from T5-1 and lacks HLA A2. The remaining HLA antigens have been confirmed to be identical with T5-1. Alloactivated cells (A+Cx)11dx produced strong suppression to Gx which shared HLA A2, B7 and B12 with the original stimulator. They also produced strong suppression to the B-cell line T5-1 stimulators which share HLA A2. However, there was hardly any suppression at all against 6.6.5 stimulators, the mutant cell line deficient in HLA A2. This experiment thus, provides formal evidence that an HLA Class I antigen, A2, can be the target of *in vitro* generated Ts.

The above data on target antigens also demonstrates that alloactivated Ts are able to inhibit the MLC response of fresh responder cells to unrelated antigens on the stimulator cells when a Ts target antigen is present on the surface of the stimulator cells. It is an interesting question of specificity as to whether the alloactivated Ts inhibit the MLC response of responder cells to unrelated antigens on the stimulator cells when the Ts come into recontact with relevant antigen on a different stimulator cell surface in the same culture as described by Rich and Rich[61,62] or only when the relevant antigens are present on the same stimulator cell surface as reported by Fitch[24] and Brondz[9].

The suppression of fresh A PBL by alloactivated cells (A+Bx)11dx to the original stimulators (Bx), unrelated stimulators (Cx), or to mixed original and HLA unrelated stimulators (Bx and Cx) is shown in Table 8. (A+Bx)11dx strongly suppressed the MLC response to the original stimulators but did not suppress the response to the unrelated stimulators. When the stimulators were mixed (Bx+Cx), the MLC of responder A to the mixed stimulators Bx + Cx was increased

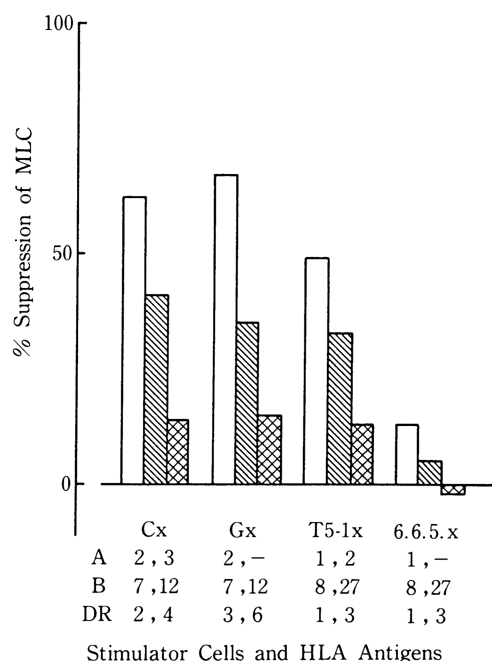


Fig. 16. PBL from individual A(HLA A11,26 B35,40 DR4,6) were primed *in vitro* against irradiated stimulator cells from individual Cx(HLA A2,3 B7,12 DR2,4). The alloactivated cells were collected on day 11, irradiated and tested for their suppressive activity in MLCs in which fresh autologous responder cells (A) were stimulated with irradiated original stimulator cells (Cx), stimulator cells (Gx) sharing HLA Class I antigens with the original stimulators, B cell line T5-1x (HLA A1,2 B8,27 DR1,3) or the mutant B cell line 6.6.5x (HLA A1,- B8,27 DR1,3) as described in Materials and Methods. The responder to stimulator to modulator ratios were 1:1:1 (▭), 1:1:1:2 (▧) and 1:1:1/4 (▩).

to 68,707 cpm. When alloactivated cells (A+Bx)11dx were added to the MLC, the reaction decreased back to 33,107 cpm which was the same response as the indicator MLC of A responding to the HLA unrelated stimulator Cx. It is conceivable that (A+Bx)11dx modulator cells had caused suppression in this assay only of the clones of responder A that were capable of responding to stimulator Bx. In order to further clarify this, experiments were made to examine the suppression of the induction of cytotoxic cells in the MLC. The results are shown in Table 9. In group I, responder A and stimulator Bx were cultured in the absence or presence of alloactivated modulator (A+Bx)11dx for seven days in a tissue culture flask and used as cytoxic effector cells. In group II, unrelated stimulator cells Dx and in group III, a mixture of original stimulators Bx and unrelated stimulators Dx were used as stimulator cells. (A+Bx)7d and (A+Dx)7d showed strong cytotoxicity for their specific target cells and hardly any cytotoxicity for the unrelated targets. However, when effector cells were induced in the presence of alloactivated (A+Bx)11dx, specific cytotoxicity to Bx was inhibited as shown in group I, but when (A+Bx)11dx was added to the culture of A responding to Dx there was no effect on the specific cytotoxicity for D targets (group II). In the case of group III, in which Bx and Dx were mixed and used as stimulators, (A+Bx+Dx)7d, as expected, showed significant cytotoxicity for both B and D targets. However, with the addition of alloactivated (A+Bx)11dx to the culture of A respond-

Table 8. Lack of Suppression of the MLC response to Unrelated Stimulator Cells in the Presence of Specific Stimulator Cells[a]

Group	Responder	Stimulator	Alloactivated Modulator	^{3}H-TdR MLC Response[b]
I	A	Bx	–	36,562 ± 2,945
	A	Bx	(A+Bx)11dx	8,916 ± 6,271
II	A	Cx	–	33,162 ± 731
	A	Cx	(A+Bx)11dx	35,937 ± 911
III	A	Bx+Cx	–	68,707 ± 2,634
	A	Bx+Cx	(A+Bx)11dx	33,107 ± 2,746

a. 125 × 10^3 responder cells were incubated with 125 × 10^3 irradiated original stimulator cells, unrelated stimulator cells as described in Table 3 or mixed irradiated stimulator cells consisting of 125 × 10^3 original and 125 × 10^3 unrelated stimulator cells.

b. Mean cpm ± 1 SD.

Table 9. Lack of Suppression by Alloactivated Ts of the Generation of Tc against Unrelated Stimulators in the Presence of Specific Stimulator Cells in MLC

Group	Responder	Stimulator	Alloactivated Modulator	Specific Cytotoxicity			
				B Lymphoblast		D Lymphoblast	
				25:1	50:1	25:1	50:1
I	A	Bx	–	45	64	9	12
	A	Bx	(A+Bx)11dx	5	8	7	15
II	A	Dx	–	3	7	40	61
	A	Dx	(A+Bx)11dx	4	6	30	40
III	A	Bx+Dx	–	31	47	35	48
	A	Bx+Dx	(A+Bx)11dx	9	11	22	35

a. 10×10^6 responder cells from A and 10×10^6 irradiated original stimulator cells from Bx, unrelated stimulator cells from Dx or mixed irradiated stimulator cells consisting of 10×10^6 Bx stimulators and 10×10^6 Dx stimulators were cultured in the absence or presence of 10×10^6 irradiated alloactivated cells (A+Bx)11dx. The cells were collected on day 7 and tested for their cytotoxicity against ^{51}Cr labelled B or D lymphoblast.

ing to both Bx and Dx together, cytotoxicity to the B target was markedly suppressed (11% at 50:1 E/T ratio), but cytotoxicity to the D target was virtually unaffected. This shows that although the suppressor antigen was present in the MLC with unrelated stimulators, the alloactivated cells inhibited the MLC response only to the stimulators cells bearing the relevant antigen, demonstrating the effector phase of suppression is also specific as well as the induction phase.

DISCUSSION

Because of our previous studies of a naturally occurring HLA B14 specific Ts cell in a bladder cancer patient[3,4,8] and the reports by others of such cells[17,18,19,20,47,51], we realized that Ts may be very important. However, such naturally occurring antigen specific Ts have been found by chance in only a limited number of persons. Thus, we felt that it is necessary to establish a convenient model system to study antigen specific Ts *in vitro* to better understand naturally occurring Ts and their role in disease. The use of the MLC system provided an opportunity to study the relationship of Th and Tc to Ts and to study immune regulation to cell surface antigens, knowledge of which might be of use in cancer therapy and organ transplantation. These were the considerations which prompted us to use the MLC as a means of studying immune regulation by Ts.

In studies of naturally occurring allospecific Ts it is common practice to add third-party modulator cells to the one-way MLC and to examine the effect of these cells on the reaction of the responder cells to the stimulator cells. In such three-cell assays, the first important point is for responder cells to react only to antigens on the stimulator cells and not react to antigens on the modulator cells. A second important point is that the added modulator cells must not react to antigens on the responder cells or stimulator cells. And a third point is that the kinetics of one-way MLC must not be changed when the modulator cells are added. A detailed analysis of the effect of added modulator cells would not be possible unless the foregoing conditions are satisfied. McMichael and Sasazuki satisfied these conditions in their study of a naturally occurring Ts[47]. They ensured that the responder cells did not react to the modulator cells by using normal responder PBL that were HLA DW2 homozygous because the subject's Ts that they used in their study were homozygous for HLA DW2. The second condition was satisfied by stopping DNA synthesis of the modulator cells by irradiation. The third condition regarding MLC kinetics was examined and found to be unchanged in their assay. Taking the foregoing points, which were developed from studies of naturally occurring Ts by Bean[4,8] and others[17,18,20,47,51] into account an assay system for MLC-induced Ts was designed.

The system we chose was patterned after that originally described by Rode and Gordon[62], the

main features of which are reviewed here. For responder cells to show no reaction to the modulator cells, the latter need to express the same HLA D locus antigens as the former. For convenience the responder cells used in Ts induction and the responder cells used in the assay are lymphocytes derived from the same donor. As evident in Table. 3, responder cells showed only a negligible reaction to 11 day autologous alloactivated irradiated cells.

Since it is known that a secondary MLC will occur when the alloactivated cells are reexposed to the original stimulating alloantigens[67], they are usually inactivated by irradiation in order to prevent their blastogenesis from occurring. We irradiated alloactivated modulator cells in the same way as was done in the three-cell assays for demonstrating naturally occurring allospecific Ts. We examined the radiosensitivity of alloactivated Ts as it had been shown by Bean and coworkers[4] that the suppressor activity of Ts in a patient with bladder cancer and by Sasazuki, Engleman and coworkers[17,47] that Ts from a multiparous woman were very radioresistant and disappeared only with irradiation of 6,000 rad. As shown in Fig. 3, alloactivated Ts are relatively radioresistant in that they exhibited suppressor activity even with irradiation to 4,500 rad. We chose to irradiate alloactivated cells with 2,250 rad because with this dose, the alloactivated cells showed no secondary MLC blastogenesis upon restimulation by specific stimulator cells (data not shown) and they exhibited strong suppressive activity. As shown in Fig. 10, the kinetics of MLC as determined in the three-cell assay with irradiated modulator cells was not altered. The three-cell assay as shown in Fig. 1 was employed for our experiments.

Although no detailed study was made concerning the duration of MLC necessary for induction of alloreactive Ts, it was confirmed that cells with suppressive activity were present by day 7 when the MLC blastogenesis and cytotoxic activity peaked (data not shown). However, alloactivated lymphocytes from 11 days of culture were used as the source of Ts for two reasons. Because lymphocytes from day 7 MLCs were at the peak of their cytotoxic activity, this cytotoxicity might interfere with interpretation of the outcome of the assay (see below). Secondly, one of the requirements discussed before was that the responder cells should not react with the modulator cells in the assay. Irradiated 7 day MLC cells autostimulate autologous fresh responder cells[10] whereas 11 day cells do not (Table 3), making the assay more straightforward in interpretation.

Another technical point of considerable importance that should not be overlooked concerns the shape of the microwells used in Ts assays. We used flat-bottom microwells in our assay, while almost all other workers used round-bottom microwells[10,16,25,27,28,65]. More cells have to be used with a flat-bottom well than with a round-bottom well in order to obtain an optimal MLC reaction. However, a flat-bottom well eliminates some artifacts because the cells do not become excessively crowded in the bottom of the microwell. In three-cell assays in particular, more cells are cultured in the wells than are usually cultured in the one-way MLC, and the degree of crowding of cells is important because the added cells are not resting lymphocytes but rather are activated lymphocytes. A flat-bottom well also offers the advantage of permitting observation under an inverted microscope to determine whether cells are activated and allows confirmation microscopically of how valid the ^{3}H-TdR uptake data are. Fig. 2 shows a comparison of MLC suppression results comparing flat-bottom wells and round-bottom wells using identical responder, stimulator and modulator cells. There is good specificity shown with the flat-bottom wells with suppression exhibited only to the original stimulator cells and none to the HLA unrelated stimulator cells. In contrast, suppression was stronger in the round-bottom wells, but the MLC reaction to the unrelated stimulator cells was also significantly suppressed. This is an important point to consider in understanding the analysis by various investigators of the target antigens and phenotypes of Ts. We have directly demonstrated here that the use of round-bottom wells can give rise to difficulties in determining the specificity or nonspecificity of suppression.

A question that has been raised since the outset of the beginning of studies on MLC induced suppressor cells is whether cytolysis of stimulator cells by alloactivated Tc rather than suppresion of MLC reaction by Ts may be the reason

for observing a decreased MLC[24]. As shown in Fig. 7, unirradiated alloactivated modulator cells used in the three-cell assay may show reactivation of Tc and produce cytolysis of stimulator cells. Further, use of alloactivated modulator cells from 7 day culture when cytotoxic activity is at its peak in MLC would, even when irradiated, probably cause some cytolysis of stimulator cells. We therefore always used irradiated alloactivated cells from day 11 of culture which have no demonstratable cytotoxicity even upon re-exposure to antigen.

Another demonstration to show that cytolysis is not the main cause of suppression is shown in Fig. 8. The extent of suppression remained almost constant in a three-cell assay in which the numbers of responder and modulator cells were kept fixed and the number of stimulator cells was increased from 31×10^3 to 500×10^3. If the inhibition of MLC was due to a cytotoxic effect by modulator cells, it should be overridden when the number of stimulator cells is increased. That is, when extra stimulator cells are present, more stimulator cells would survive and stimulate proliferation. Sheehy, Mawas and Charmot[68] performed the same types of experiments and observed the same results as these. Additional evidence against Tc being the cause of the suppressed MLCs has been provided other workers. Gressor and his colleagues[25] demonstrated that when the primary MLC was performed in the presence of interferon, there was good generation of Tc. However, when these alloactivated cells that possessed strong cytotoxicity were used as a modulator cells in the MLC of original responding cells with the appropriate stimulator cells, suppression was nearly absent.

From a different approach, Hess et al[37] found that cells showed no cytotoxicity but did show good suppressor activity when the primary MLC was conducted in the presence of CsA which inhibits induction of cytotoxic cells in MLC. It was also confirmed that MLC suppression was unaffected by CsA (Fig. 9). However, the final direct proof that elimination of stimulator cells is not involved in the mechanism of MLC suppression must await experiments using a cytotoxic cell clone and a suppressor cell clone derived from the same MLC and the demonstration that the cytotoxic clone shows cytotoxicity but no suppression and that the suppressive clone shows suppression but no cytotoxicity.

The 11 day MLC primed cells which had allospecific suppressive activity were almost 100% T cells, and two-thirds of them expressed HLA DR antigens (Fig. 11 and Table 6). These findings are similar to the results on activation of cells in culture reported by other workers[16,26]. The fluorescence pattern of cells stained with anti CD2 in which dull-stained cells are decreased in number and bright-stained cells are increased very closely resembles the pattern seen with peripheral blood T cells of the patient with anti-HLA B14 Ts reported previously[8]. We have also conducted tests to characterize the cell surface phenotype of these MLC generated suppressor effector cells. Although the suppressor activity disappeared almost completely when the alloactivated cells were treated with monoclonal antibody anti CD8 and complement, some suppression remained at high concentrations of modulator cells (Fig. 12). However, as the fluorescent pattern of cells stained with anti CD8 (Fig. 11) shows a large number of dull-stained $CD8^+$ cells were present in the day 11 alloactivated cells which would be difficult to eliminate completely by anti CD8 and complement treatment. This was probably the cause of the weak remaining suppression as the results obtained by panning with $CD4^+$ and $CD8^+$ cells clearly demonstrated the presence of suppressor activity only in $CD8^+$ cells (Fig. 13). In numerous tests we failed to detect any suppression at all by positively selected $CD4^+$ cells. This finding is in contrast to the reports of Damle et al[10] and of Goeken and her colleagues[28]. In the study of Goeken et al, isolated $CD4^+$ and $CD8^+$ subsets were stimulated in allogeneic MLC and subsequently tested for their ability to suppress fresh autologous responding cells in a fresh MLC. They found both $CD4^+$ and $CD8^+$ cells showed suppression. In another series of experiments, $CD4^+$ and $CD8^+$ cells were also separated from previously alloantigen primed T cells. Both alloactivated $CD4^+$ and $CD8^+$ cells suppressed the MLC. They concluded that the precursor and effector of Ts existed in both $CD4^+$ and $CD8^+$ subsets[28]. However, it was not clear whether the suppression was antigen specific or not, because they presented no data on specificity controls using HLA unrelated third

party stimulator cells. And, in contrast to our study, they used round-bottom microwells. Therefore, they may have detected in both the $CD4^+$ and $CD8^+$ cell subsets, the kind of nonspecific suppressor activity of activated lymphocytes similar to that induced by mitogens[21,57,69]. In fact, they demonstrated different kinetics in the one-way MLC when nonirradiated activated T cells were added to the MLC.

Engleman's group also demonstrated both $CD4^+$ and $CD8^+$ cells from alloprimed cultures produced suppression of fresh MLCs[10]. In further studies they demonstrated that $CD8^+$ Ts effector cells can apparently be induced by $CD4^+$ suppressor/inducer cells. In their studies, $CD8^+$ or $CD4^+$ cells plus accessory cells were cultured with irradiated allogeneic stimulator cells for 7 days. Fresh $CD8^+$ cells responded to the irradiated autologous alloactivated $CD4^+$ cells but not the activated $CD8^+$ cells in the absence of allostimulator cells. Interestingly, $CD8^+$ cells that had been stimulated with irradiated alloactivated $CD4^+$ could suppress the MLC of fresh $CD4^+$ cells responding to the original stimulators. The suppressive influence of such $CD8^+$ cells was dose-dependent and specific. They concluded HLA DR antigens on the alloactivated $CD4^+$ cells might play a role in the activation of $CD8^+$ Ts, because these $CD8^+$ Ts had not been directly exposed to either HLA Class I and/or Class II antigen on the stimulator cells. They did not report data on the suppressor cell phenotype or the suppressive activity of PBL or subsets simply cultured with stimulator cells and then tested for activity as performed here. It is conceivable that these different experimental protocols might induce different Ts phenotype. For example, an alternative explanation for what Damle et al[10] have observed and briefly discussed would be that they have produced an anti-idiotype (anti-clonotypic) suppressor reaction like that described by Lamb and Feldman[45] rather than generating $CD4^+$ suppressor inducer cells which induced $CD8^+$ suppressor effector cells. That is, the initial culture of allostimulator cells would expand $CD4^+$ cells having T cell receptors (clonotypic) for alloantigen. Use of these primed $CD4^+$ cells as stimulators of fresh autologous $CD8^+$ cells in the absence of alloantigens in the secondary culture would allow the $CD8^+$ cells to be exposed to stimulatory levels of the T cell receptor idiotype and become anti T cell receptor idiotype Ts.

It has been reported that, with the use of monoclonal antibody 9.3 with which $CD8^+$ cells can be divided into two groups, cytotoxic precursors were found to be present in the $CD8^+$ 9.3^+ population and suppressor precursors in the $CD8^+$, 9.3^- population[12]. 9.3 reacts with approximately 75% of peripheral T cells including almost all $CD4^+$ cells and 30-50% of $CD8^+$ cells[33]. Engleman's group[12] cultured $CD4^+$ cells and autologous $CD8^+$ 9.3^+, or $CD8^+$ 9.3^- cells with irradiated stimulator cells for 7 days. Then, the activated $CD8^+$ cells were recovered by panning with anti CD8 and examined for their ability to lyse allogeneic target cells. Alloactivated $CD8^+$ 9.3^+ cells lysed targets cells of both the original stimulator and of third party cells sharing HLA Class I antigens, but not third party cells sharing only HLA Class II antigens. In contrast, the $CD8^+$ 9.3^- population showed no cytotoxicity at all. In the suppressor assay, however, alloactivated $CD8^+$ 9.3^- cells suppressed the proliferative response of fresh $CD4^+$ cells to the original stimulator cells. The suppressive effects of $CD8^+$ 9.3^- cells were specific for the HLA DR antigen of the stimulator cells. On the other hand, $CD8^+$ 9.3^+ cells only weakly inhibited the MLC. They concluded that this weak inhibitory effect of $CD8^+$ 9.3^+ cells might be due to a cytotoxic mechanism. If so, $CD8^+$ 9.3^+ cell should inhibit the MLC against HLA Class I antigens shared third party cells. But, they did not demonstrate the inhibitory effect of alloactivated $CD8^+$ 9.3^+ cell on the proliferation to HLA Class I sharing stimulator cells. Therefore, it is still unclear whether this Class I specific suppression was due to a cytotoxic mechanism. And although we have found suppressor effector cells in the $CD8^+$ 9.3^+ population, no conclusion can be made if this phenotype is the same or different since the report of Engleman et al presents no data regarding the effector phenotype and we have not studied the precursor phenotype.

Our study of the specificity of suppression has clarified that it is possible to induce Ts *in vitro* which are specific to HLA Class I antigens and to HLA A2 in particular. Many reports[10,11,16,25,65,66] describe that the Ts produced

using similar culture conditions as that used by us and other workers are specific to HLA Class II antigens of the stimulator cells and to DR antigens in particular. It was Sheehy et al[68] who first demonstrated the potential presence of Ts specific to HLA Class I antigens when they showed the specificity of these alloactivated cells for both HLA Class I and/or Class II antigens. Goeken and co-workers observed similar results[27]. However, no other investigators have as yet definitively shown the presence of alloactivated Ts specific to HLA Class I antigens. We clearly demonstrated in an experiment using the B cell line T5-1 (HLA, A1, A2, B8, B27, DR1, DR3) and the variant cell line derived from it 6.6.5 (HLA, A1, B8, B27, DR1, DR3) lacking A2 that alloactivated cells specific to HLA A2 antigen suppressed the proliferative response to T5-1 but not 6.6.5 (Fig. 16).

The significance of finding that suppression can be triggered by HLA Class I is very great. A review of the literature concerning naturally occurring allospecific Ts shows that such Ts are all apparently Class I specific[3,4,8,18,20,47]. The prototype example are the Ts that came from a multiparous woman[18,47]. These Ts inhibited the response to allostimulators only when the stimulator cells bore HLA BW35 or closely related antigens. That is, the BW35 antigen(s) were recognized by the Ts. The naturally occurring Ts cells reported by Fainboim et al had suppressor activity to stimulator cells having BW35[20]. The naturally occurring Ts reported by Bean et al were specific to HLA B14[3,4,8]. In view of the above, naturally occurring allospecific Ts are probably triggered by HLA Class I antigens to produce inhibition of MLC. Hence, the production of HLA Class I specific Ts in our *in vitro* model is not incongruous and may make it a more relevant biological model.

The view that suppression specific for HLA Class II antigens is a true phenomenon of suppression caused by Ts and the suppression specific for HLA Class I antigens is a phenomenon occurring as a result of elimination of stimulator cells by Tc is contradictory. Although it is well-known that MLC-induced Tc are specific mainly for HLA Class I antigens, analysis using clones has revealed the presence of Tc specific to HLA DR[43]. There is no reason why DR specific Tc are not induced by MLC which induces DR-specific Ts. We have shown that alloactivated Ts are specific for both HLA Class I and HLA Class II antigens. Therefore, it is hard to believe that only those instances of HLA Class I specific inhibition of MLC are due to elimination of stimulator cells.

To determine the range of antigens that could be targets of Ts we used the lymphocytes of two donors as responder cells and stimulated them with the same HLA unrelated stimulator cells. Then we examined the suppression of the response to various stimulator cells sharing only one known HLA antigen with the original stimulator cells (Fig. 5). Responder cells from individual A showed good suppression to stimulators having A2, B7 and DR2 in common with the original stimulator cells, but only weak suppression to HLA B12. Q responder cells, however, showed no suppression to HLA A2, B7, B12 and DR3 although they showed suppression to DR2. This was a very interesting observation, which showed that there are antigens to which suppressor cells are easily induced in any given responder and antigens to which suppressor cells are not readily induced. A possible explanation of this observation is that a difference of genetic background of responder cells might affect induction of Ts. In the murine model, Benacerraf et al demonstrated that there were non-responder and responder strains of mice to GAT, a linear synthetic polymer, and that in non-responder mice, active suppressor cells were induced by GAT stimulation[40]. This discovery became the basis for the subsequent clarification of the genetic control of immune response[13], suppressor mechanisms[15] and suppressor factors of Ts[71]. Genetically controlled Ts in humans were reported by Nishimura and Sasazuki[51], who discovered that there were high responders and low responders to streptococcus cell wall (SCW) antigens and that there were genetically controlled Ts among the low responder cells. It is very interesting that lymphocytes of individuals with different genetic background should show such a different responsiveness to the same antigens. However, a detailed family study and study of their HLA antigens would have to be made in order to derive a conclusion as to whether or not our alloactivated Ts are genetically controlled.

MLC-induced Ts inhibit the MLC of fresh

responders when they come into re-contact with the priming antigen. It is a very interesting question as to whether the suppressor target antigen induces the Ts to inhibit the responder cells nonspecifically or whether the Ts inhibit the response only to stimulator cells bearing the appropriate antigen. Thus, a study was made on how Ts affected responses to mixed stimulators by mixing the original stimulator Bx cells which elicit suppression and unrelated stimulator cells Cx which do not trigger suppression. As a result, Ts (A+Bx)11dx showed 52% suppression of the MLC of mixed stimulator cells of Bx and Cx and reduced the MLC blastogenesis to the same level as A+Cx with or without (A+Bx)11dx modulator cells (Table 8). However, it was not definitive in this experiment whether the MLC to the original stimulator cells, the MLC to the unrelated stimulator cells, or the MLC to both was inhibited by allospecific Ts. Therefore, a study was made of the suppression of the generation of cytotoxicity in MLC using the appropriate stimulator cells as target cells. It was found that whereas cytotoxicity to the original stimulator cells among the mixed stimulator cells was strongly inhibited by Ts, cytotoxicity to the unrelated stimulator cells was hardly inhibited at all. This showed that Ts inhibited the MLC reaction to only the original stimulator cells and did not inhibit reactions to unrelated stimulator cells. This phenomenon has also been reported in murine models[9,24]. In one such study[24], allostimulated cells (A + C3Hx) 6dx inhibited the generation of Tc to target cells from the C3H strain but not to target cells from the unrelated DBA/2 strain when MLCs were performed with A responding to mixed stimulator spleen cells from C3H and DBA/2 strains. However, (A + C3Hx)6dx inhibited the generation of Tc to target cells from both C3H and DBA/2 strains when C3H and DBA/2 F1 hybrid spleen cells were used as stimulator cells in the suppressor assay instead of a mixture of stimulators from C3H and DBA/2. Thus the following hypothesis must be true (Fig. 17): Ts cannot inhibit the reaction of responder cells to an unrelated alloantigen unless the Ts triggering antigen is on the surface of the same cell as the unrelated alloantigen. The naturally occurring Ts cells triggered by HLA BW35, which were reported by Sasazuki et al[17,47] and Fainbloim et al[20] as well as the data presented here can be satisfactorily explained by this model.

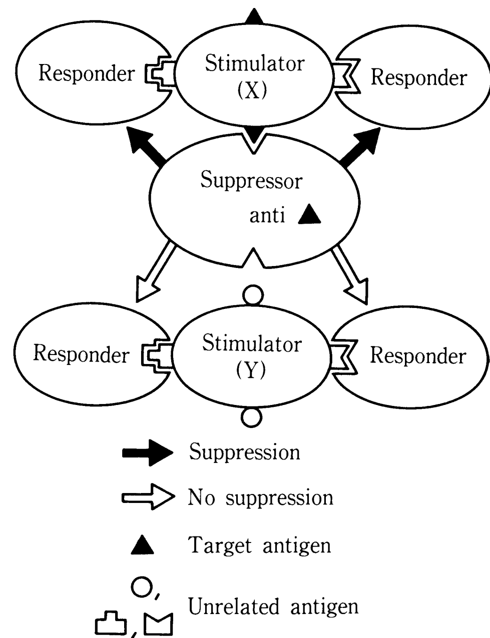


Fig. 17. Schematic representation of the action mode of suppressor T cells.

This immunologic mechanism may well be the explanation for apparent immunosuppression to non-tumor related antigens present in some patients with cancer. As the tumor grows, it may stimulate tumor specific Ts cells to either tumor antigens or normal cell surface molecules to which we are tolerant. As the tumor progresses in size and sheds soluble antigens and complexes into the system, these antigens may bind to antigen presenting cells in the skin and other organs of the body. Thus, when the patient is challenged with an antigen such as DNCB or PPD, this non-tumor related antigen would be seen by the immune system in close proximity with the antigen that is inducing the suppressor cells. That is, the immune response to the unrelated antigen would be blocked since it is seen on antigen presenting cells already displaying the relevant suppressor inducer antigen.

These basic studies of MLC-induced Ts should provide us with an understainding of the mechanism of the beneficial effect of donor specific blood transfusion in kidney transplanation. It is known that the graft survival rate increases for two thirds of the patients if blood

transfusion from the organ donor is given prior to transplantation[64], which strongly suggests the possibility that Ts are being induced. However, one fourth to one third of patients become sensitized and do not receive the proposed kidney[64].

The existence of HLA Class I specific Ts definitively demonstrated in this paper is relevant to organ transplantation, because HLA Class I antigens are expressed on both nucleated blood cells and transplanted organs, whereas HLA Class II antigens are present primarily on monocytes and B lymphocytes in the blood. HLA Class I specific Ts induced in a recipient of an organ graft would inhibit immune reactions such as cytotoxic cell generation and cytolytic antibody production to the cell surface antigens on the transplanted organ. If we could detect the most appropriate antigen(s) for induction of Ts in the recipient from the *in vitro* tests using this MLC induced suppressor system, it would provide a useful method for us to prescreen the donor-recipient combinations and pick the ones that preferentially induce suppressor cells.

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