# HLA Typing: Methodology and Clinical Aspects

## Volume II

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Chapter 10

# HLA-A,B RESTRICTION OF CYTOTOXIC T CELLS

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## I BACKGROUNDS AND MODELS

### A Backgrounds

The availability of an in vitro technique for the induction and differentation of specific cytolytic effector T cells has made a significant contribution to the understanding of the role of the Major Histocompatibility Complex (MHC) antigens in the immune response, and provided as in vitro model of the homograft reaction. Hirschhorn and co workers[1] were the first to demonstrate that human peripheral blood lymphocytes (PBL) cultured with human fibroblasts from unrelated individuals were able to lyse allogeneic fibroblasts. Hayry and Defendi[2] described that mouse lymphocytes sensitized in vitro with allogeneic lymphocytes were able to destroy only the lymphoma line target cells which carried the specific sensitized antigens and did not affect cells which were isogeneic to the original responding cells. In man, Solliday and Bach[3] showed preferential destruction of lymphoblastoid cell lines isogeneic to the sensitizing cell, although cross reactivity with other cell lines was observed. The so called Cell Mediated Lympholysis (CML) technique was subsequently developed by Lightbody et al.[4] The technique consists of two in vitro phases: an induction phase in which lympholytic effector cells are induced, and an effector phase in which the effector cells lyse chromium labeled target cells. The generation of effector cells in the first step is necessary in order to obtain measurable cytotoxicity in the second step.

A direct CML, so called LMC (Lymphocyte Mediated Cytolysis), consists of only one in vitro step. PBLs, removed from, for example, an in vivo sensitized patient are tested without an induction phase directly against chromium labeled target cells.

The CML technique was originally used to study the genetics of the MHC. The determinants recognized by that technique are in all probability class I (HLA A, B, C) specificities[5-7] but also class II (HLA D) antigens (Mawas et al.[8] Leighery et al.[9] and Albrechtsen et al.[7]) or determinants closely linked to them. They have also been referred to as CD determinants or cytotoxic defined determinants.

Zinkernagel and Doherty[10] used the CML technique to study the specific interaction of virus and MHC determinants which has been of major importance for our understanding of the immune response in general and the role of the MHC determinants in particular. The observed phenomenon was called MHC restriction and was first studied by them in the mouse in a model of virus induced lymphocytic choriomeningitis soon followed by a model of hapten MHC restriction (Shearer[11]).

The H-2 restriction phenomenon was not limited only to virus and chemically altered cell surface products. Bevan[12] demonstrated H-2 restricted cytotoxic T cells directed towards non H-2 minor histocompatibility antigens. Gordon et al.[13] showed the involvement of the H-2 region products to obtain cytotoxic T cell response against the male specific antigen, H-Y.

The mechanism by which these H-2 restricted cytotoxic T cells recognize modified self surface products still remains unclear. At least two possible models (the dual and

single recognition model) have been proposed [4] More recently experiments by Bur akoff et al [15] may suggest that cytotoxic T cell responses against alloantigens may be compatible with responses against autologous MHC products modified for example by viruses

## B Models

### *1 HLA Restricted Anti H Y Cytotoxicity*

The involvement of the MHC in T cell mediated HLA restricted cytotoxic response against foreign in rodents was confirmed in man and has been detected for a minor histocompatibility antigen namely the male specific antigen H Y (Goulmy et al ) [6] In this example the primary sensitization occurred in vivo PBL of a bone marrow transplanted female aplastic anemia patient showed direct and indirect cell mediated cytotoxicity only against male target cells which carried one of the HLA antigens of the original sensitizing cell namely the HLA A2 antigen This antigen was also present on the patient s lymphocytes

This observation of HLA A locus product restriction of cytotoxic T cells directed against the minor histocompatibility antigen H Y was later confirmed in four other cases (all multitransfused female aplastic anemia patients) The subsequent cases dem onstrate also that the same magnitude of H Y immune cytotoxicity could be observed in conjunction with HLA determinants coded for by different haplotypes (Goulmy et al [7]) Experiments by Singal et al [17a] have confirmed the killing of HLA A2 positive male target cells by in vivo immunized HLA A2 positive females They showed that pregnancy in itself is sufficient to induce sensitization to HI A restricted cytotoxicity

### *2 HLA Restricted Anti Virus Cytotoxicity*

#### *a Epstein Barr Virus (EBV)*

Tursz et al [18] have suggested that HLA A and B region determinants are necessary for anti viral T cell mediated cytotoxicity towards EBV sensitized T cells They re ported that EBV sensitized peripheral T lymphocytes from patients with infectious mononucleosis sensitized against EBV failed to lyse EBV infected Daudi cells which apparently lack the HLA class I antigens at the membrane but could lyse all other EBV cell lines without any apparent HLA specificity On the other hand no evidence for allogeneic restriction in this system was apparent [6a] Lipinski et al [8] suggested that the HLA region appears to act at two different levels in the T cell mediated lysis of EBV infected cells Experiments by Rickinson et al [9] confirmed that the HLA A and B region products indeed play a role in the T cell recognition of EBV infected B cells A recent report by Misko et al [9a] strongly indicated that T cell cytotoxicity to EBV is restricted by HLA antigens In this (in vitro) study 14 days cultures of lympho cytes from EBV seropositive donors have been investigated and specificity was dem onstrated using EBV infected lymphoblastoid cell lines as target cells

#### *b Influenza Virus*

McMichael and Ting [20] who showed that cytotoxic T lymphocytes and influenza virus infected target cells must share a part cular HLA antigen Cytotoxic effector cells specific for one type of influenza virus A/X31 or B/Hong Kong killed the autologous and HLA matched infected target cells but not HLA mismatched cells HLA B7 seemed to be the required restricting antigen The HLA A2 antigen seemed to be in adequate in the cytotoxic T lymphocyte recognition of influenza virus McMichael[21] and Biddison et al [22] have confirmed these findings However both studies reported discrepancies between the serologically defined HLA specificities and the determinants which are recognized by the T cells in association with the influenza virus The latter authors discussed the possibility that T cells may even discriminate between the sero

ogically defined HLA antigens and those that are not yet serologically distinguishable.[23] Furthermore, Shaw and Biddison[24] investigated families to study the genetic control of the in vitro T cell responses to influenza virus-infected autologous cells and found responses that were associated with preferential haplotypes. The results of studies using unrelated donors[25] demonstrated variable lytic capacity to virus infected self target antigens. The degree of responsiveness could be explained on responder, stimulator, or target level. The peculiar behavior, i.e., different cytotoxic T cell responses towards virus-infected target cells sharing the same HLA antigens, could be explained by a genetically controlled regulation system (Biddison and Shaw).[25] These studies have been extended, with observations showing preferential recognition of HLA target antigens for the different, but closely related, influenza types A/Hong Kong and B/Hong Kong (Shaw et al.).[26]

*c. Measles Virus*

Another example of the HLA restricted cytotoxic T cell function was found in association with measles virus (Kreth et al.).[27] The lymphocytes of five patients with acute measles preferentially lysed measles virus-infected target cells which shared the appropriate HLA-A or -B determinants. Apparently no restriction is found after the acute phase of the disease.

*d. Herpes Simplex Virus (HSV) and Human Cytomegalo Virus (HCMV)*

Studies by Sethi et al.[28] demonstrated HLA restriction in the lytic activity towards HSV and HCMV infected skin fibroblast target cells. The presence of virus-specific cytotoxic lymphocytes (CTLs) was shown by using long-term cultures derived from peripheral blood lymphocytes from in vivo sensitized patients. A recent report by Quinan et al.[28a] demonstrated the development of HLA-restricted CTL in vivo in four bone marrow transplant recipients during acute CMV infection. Earlier attempts to demonstrate HLA restricted cytotoxic T cells during primary infections have failed; Steele et al.[29] rubella virus; Perrin et al.[30]: vaccinia virus; Perrin et al.[31]: measles virus.

*3. HLA Restriction of Chemically Modified Cells*

*a. Trinitrophenyl (TNP)*

The first attempt to demonstate the involvement of HLA restriction in a hapten modified system was described by Newman et al.[32] Specific cytotoxic lymphocytes (CTLs) elicited responses to only the TNP-coupled autologous cells. However, there was an absoute need for boosting after in vitro priming. Friedman et al.[33] reported, in a more extensive study, that the cytotoxic activity was mediated by T cells, and could be triggered in vitro. Restriction to TNP modified 'self' but not for unaltered or TNP-modified allogeneic target cells was described. Shaw et al.[34,35] have demonstrated that cytolytic activity could be observed not only against TNP-modified autologous cells but also against modified allogeneic cells. They also observed differences in donors' responses to the same modified allogeneic self antigens. The presence of the polymorphic MHC determinants such as the HLA-A and -B locus antigens was not an absolute requirement for the human cytotoxic T cell responses to TNP-modified target cells. These authors suggested that the anti TNP T cell recognition responses could be associated with multiple recognition structures of the cell surface determinants. Charmot and Mawas[36] have confirmed Shaw's findings that MHC restriction, in the TNP Model, is not a general phenomenon. In addition, Seldin and Rich[37], Charmot and Mawas[36] and Friedman et al.[38] reported the requirement of HLA-D region products to trigger specific TNP-cytotoxic T cells in their response to TNP-modified autologous target cells.

*b Dinitrofluorbenzene (DNFB)*

The hapten DNFB has been used to study whether or not lymphocytes from Dinitrochlorobenzene (DNCB) sensitized patients were able to produce cytotoxic responses against DNFB treated autologous and HLA compatible target cells (Dickmeiss et al [39]) The observed cytotoxicity was directed against only DNFB coupled target cells and was seen (in three cases) in conjunction with the HLA-A2 antigen

*c Fluorescein Isothiocyanate (FITC)*

Friedman and his colleagues[40] also used FITC as an hapten for the generation of cytotoxic T cells to FITC-conjugated autologous target cells In addition, they demonstrated the inability of cytotoxic T cells specific for FITC to lyse TNP modified autologous target cells, thus indicating the hapten specific altered self reactivity The role of MHC determinants in this system is still unclear

## II TECHNICAL ASPECTS

### A Cell Mediated Lympholysis (CML) Assay

The method that is currently used in our laboratory to detect cytotoxic T cell responses to the minor histocompatibility antigen (H Y) will be described

*1 Induction or Sensitization Phase*

*a Preparation of the Effector Cells*

Blood is collected into sterile bottles containing preservative free heparin (50 IU/ml)

The lymphocytes are separated by Ficoll Ispaque gradient centrifugation

Cell concentration responder cells $1 \times 10^6$ cell/ml, stimulator cells $1 \times 10^6$ cell/ml (inactivated by 2000 rad $\gamma$ irradiation) Depending on the amount of cell recovery, 50 ml culture flasks or 2 ml cluster wells are used The culture flasks are stored at a 45° angle during the culture period

Culture conditions and duration 37°C, 5% $CO_2$, well humidified incubator, 144 hr

Culture medium RPMI 1640, supplemented with 3 m*M* L glutamine 100 IU penicillin/ml, 100 µg streptomycin/ml and 20% heat inactivated pooled human AB serum from male donors

Washing fluid Hanks' BSS supplemented with 50 IU penicillin/ml

*b Preparation of the Target Cells*

Cell concentration 5 to $8 \times 10^6$ cells are cultured in 5 to 8 ml culture medium Phytohemagglutinin (PHA) is added for the culture duration of 72 hr

Additional note fresh or 6 day cultured unstimulated lymphocytes can also be used as target cells although lower percentages of lysis will be obtained in the effector phase An example (with special regard to the specific HLA A2 restricted anti H Y lysis) of the use of stimulated and/or unstimulated target cells is shown in Figure 1

*2 Destructive or Effector Phase*

*a Preparation of the Effector Cells*

After the 6 day induction phase, the effector cells are transferred to 50 ml tubes, centrifugated at 350 × g for 10 min, resuspended in 1 to 2 ml fresh culture medium Viability counting is performed with eosin 0 1% Thereafter the cells are brought to the desired concentration (see c)

*b Preparation of $^{51}$Cr-Labeled Target Cells*

The target cells are gently collected from the flasks and transferred to 15 ml conical bottomed tubes Centrifuged at 150 × g for 10 min The supernatant is decanted and the target cells are resuspended in the remaining 0 4 ml supernatant

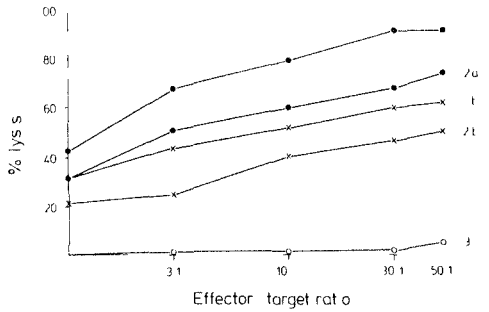


FIGURE 1 Specific lytic capacity against two HLA A2 positive mitic target cells (comparison between blasts and PBLs) 1a HLA A2 ♂ target cells (blasts) 1b HLA A2 ♂ target cells (PBLs) 2a HLA A2 ♂ target cells (blasts) 2b HLA A2 ♂ target cells (PBLs) 3 HLA A2 ♀ target cells (blasts) Percentage lysis expressed in LU 50 1a 1 4 2a 2 8 1b 7 6 2b 50 3 >100

Labeling 100 $\mu$Ci $Na_2$ $^{51}CrO_4$ Amersham CJS IP 5 mCi/5m$\ell$ spec act 100 to 350 mCi/mg

Incubation 1 hr in a waterbath at 37°C After incubation approximately 4 m$\ell$ washing fluid is added gently mixed and centrifuged at 150 × g for 10 min The pellet is washed twice and thereafter resuspended in culture medium

Viability counting is performed with eosin 0 1% The cells are brought to a concentration of $1 \times 10^5$/m$\ell$

c *Assay*

Add 0 1 m$\ell$ effector cell suspension and 0 1 m$\ell$ target cell suspension to each well of a round bottomed microtiter plate Preferential effector cell target cell ratios are 50 1 25 1 5 1 All assays are performed in triplicate

Microtiter plates with targets and effectors are centrifuged for 2 min at 150 × g then incubated for 4 hr at 37°C

Spontaneous release 0 1 m$\ell$ target cell suspension and 0 1 m$\ell$ culture medium/well (in triplicate) are incubated in a microtiter plate for 4 hr at 37°C

Maximum release (in triplicate) 0 1 m$\ell$ target cell suspension and 0 1 n$\ell$ of a Zaponine solution (10 m$\ell$ RPMI 1640 + 10 drops Zaponine Coulter Electronics Ltd ) are incubated for 4 hr at room temperature

*3 Harvesting and Counting*

All microtiter plates are centrifuged for 5 min at 500 × g The supernatants are removed with the Flow supernatant harvester (the titertek system) (The use of this titer tek system is originally described by Hirschberg et al [44]) The samples are counted for 1 or 2 min in a γ counter (Packard 5260)

*4 Calculation and Interpretation*

The percent lysis is calculated using the following formula

$$\frac{\text{Experimental mean cpm} - \text{spontaneous release mean cpm}}{\text{Maximum release mean cpm} - \text{spontaneous release mean cpm}} \times 100$$

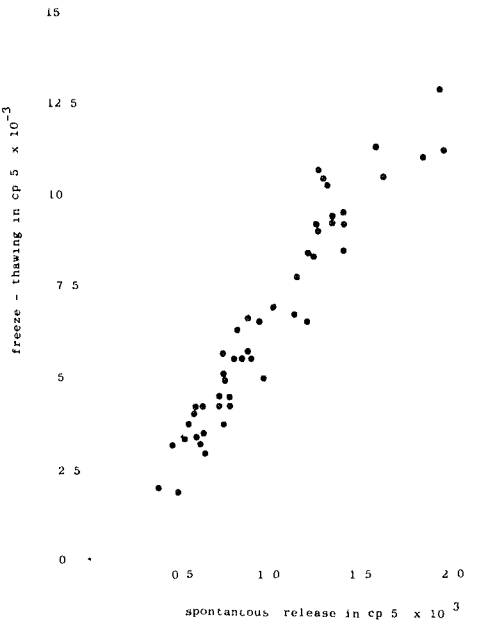


FIGURE 2 Relationship between spontaneous and maximum release value

Standard errors of the means of triplicates has to be less than 5% The results are expressed on a scale in which the spontaneous release value is set to 0% and the maximum release value to 100% (see Figure 2) Spontaneous release which is determined in wells with assay medium alone, normally amounts to 15 to 25% of the maximum release

When only one effector to target (E/T) ratio is used, lysis percentages equal or below 10% are considered as negative, 11 to 15% as weakly positive, 16 to 40% as positive, and >40% as strongly positive When the number of cells for an experiment are limited, positive and negative assignments are made on the basis of a 10% specific Cr release value This criterion is used also for the assignment CML positivity by the European CML study group The expression of percentage lysis in lytic units (LU) is a useful parameter of CML activity LU 50, for example, is the number of effector cells $\times 10^{-4}$ necessary to obtain 50% specific lysis of $10^4$ target cells According to this definition, a larger number of LUs reflects a weaker response because more effectors are required to cause a 50% CML response

Our laboratory participated in a collaborative study to standardize the CML technique The results of four CML workshops and a recommended European standard CML technique have been reported [41 44]

### B Monolayer Absorption Technique

A monolayer absorption technique has been used to test whether killer cells directed to self HLA associated with the minor histocompatibility antigen (H-Y) were divisible into subsets of killer T cells (Goulmy et al [17])

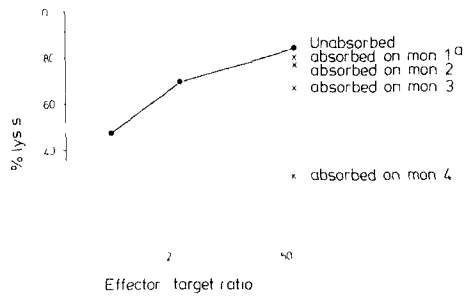


FIGURE 3 Absorption of the HI A A2 restricted anti H Y cyto toxic effector cells Monolayer 1 HI A A2 ve ♂ monolayer 2 HI A A2 – ve ♀ monolayer 3 HLA A2 ve ♀ monolayer 4 HI A A2 + ve ♂ The unabsorbed and absorbed CTLs are tested on HI A A2 positive male target cells

This technique which allows the partition of cell populations was developed in our laboratory by Hamilton et al [45] based on monolayer absorption studies done by oth ers [47 49]

*1 Preparation of the Monolayers*

Monolayers were prepared from fresh PBL s (resuspended in serum free medium in a concentration of $20 \times 10^6$ cells/m$\ell$) adhering to Petri dishes (35 × 10 mm) which are treated with a 50 $\mu$g/m$\ell$ solution of Poly L Lysine (PLL mol wt 230,000 Sigm a Chemical Co St Louis, Mo ) After incubation for 1 hr at room temperature the nonadherent cells were removed and the effector cells (adjusted to $10 \times 10$ cells/m$\ell$ in RPMI 1640 with 20% pooled human serum) were gently overlaid on the washed monolayer and incubated for 1 hr at 37°C Thereafter the supernatant and the nona dherent cells were decanted and tested at desired concentrations for specific cytolytic reduction

*2 Results and Interpretation of Anti H Y Killer Cell Activity*

Killer cells directed against "self" HLA associated with the minor histocompatibil ity antigen H Y can be specifically absorbed by the monolayer Furthermore they can be partitioned into separate populations recognizing different altered self HI A gene products (Goulmy et al [17])

***a Depletion in Cytotoxicity of the HLA-A2 Restricted Anti H-Y Cytotoxic T Cells***

Figure 3 shows the results of the reduction in lysis after absorption of the HLA A2 restricted anti H Y cytotoxic effector cells by the appropriate monolayer (i e the HLA A2 positive male monolayer)

***b Reduction in Cytotoxicity of the HLA-A2 and HLA-B7 Restricted Anti H Y Cyto toxic T Cells***

Figure 4 shows the independent absorption of both (A2 H Y and B7 H Y) subsets of cytotoxic effector cells Complete removal of the HLA A2 restricted anti H Y cy totoxicity (see Figure 5) was observed when the effector cells (possessing cytotoxic activity directed against the two independent phenotypes) recovered from an HLA A2 positive male monolayer were tested against HLA A2 positive male target cells The

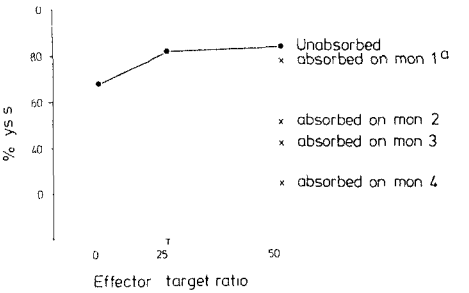


FIGURE 4 Independent absorption of the anti HLA A2 H Y and anti HLA B7 H Y cytotoxicity Monolayer 1 HI A A2 – ve B7 – ve ♂ monolayer 2 HI A A2 + ve B7 ve ♀ monolayer 3 HLA A2 ve B7 + ve ♂ monolayer 4 – HLA A2 + ve B7 + ve ♂ The unabsorbed and absorbed CTLs are tested on HLA A2 positive B7 positive male target cells

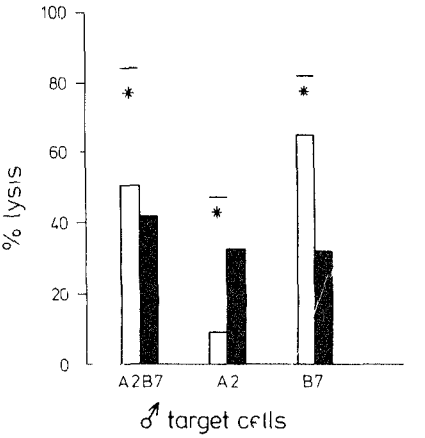


FIGURE 5 Reduction in specific cytotoxicity after monolayer absorption —— unabsorbed □ monolayer HI A A2 + ve ♂ ■ monolayer HI A B7 + ve ♂ ✱ monolayer HLA A2 ve B7 ve ♂

converse effect was seen after absorption of the effector cells on an HLA B7 positive male monolayer thereafter an almost complete depletion of the HLA B7 restricted anti H Y cytotoxicity was observed when the effector cells were tested against HLA B7 positive male target cells (Figure 5)

Partial reduction in lysis was observed after absorption with either an HLA A2 or an HLA B7 male monolayer and thereafter tested against target cells which carried *both* restricting HLA antigens

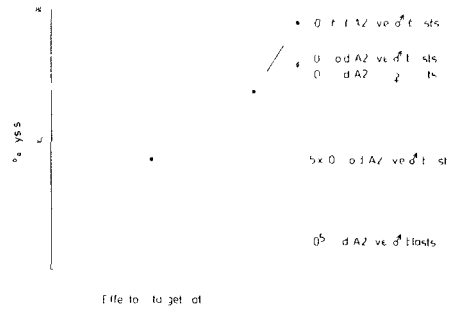

FIGURE 6 Reduction in specific cytotoxicity by addition of unlabeled target cells to the labeled target cells

The latter results provide evidence that the anti B7 population can be absorbed independently of the anti A2 population. Effective reduction was also seen after absorption on a monolayer carrying both restricting antigens, A2 and B7 (Figure 4). The removal (by monolayer absorption, Figures 3, 4, and 5) of specific subpopulations of restricted effector cells may be compatible with the hypotheses of altered self or dual recognition (Zinkernagel et al.)[14] However, more functional assays are required to resolve the question.

### C. Target Cell Inhibition Studies

Unlabeled target cells are able to compete with labeled target cells provided that they express similar antigenic membrane determinants (CML blocking technique[50-53]).

Cold target inhibition studies were performed to confirm the monovalent specificity of the HLA restricted anti H-Y cytotoxic T cells. The advantage of the monolayer absorption technique is that subpopulations can be physically removed from cell suspensions. However, (with special emphasis on effector cells recognizing two "altered" self HLA specificities) cold target inhibition studies result in competitive inhibition during the cytotoxic T cell target cell interaction and theoretically cannot distinguish between polyvalent and monovalent effector cells.

#### *1. Cold Target Inhibition of the HLA-A2 Restricted Anti H-Y Cytotoxic Cells*

Figure 6 shows the specific reduction in cytotoxic activity when $1 \times 10^5$ cold HLA-A2 positive male blast cells have been added to the hot targets. Furthermore, Figure 7 shows that the specific HLA-A2 H-Y lysis does not cross react with the serologically cross reacting HLA-A28 (no inhibition). Unstimulated lymphocytes (PBLs) are able to inhibit but a 10-fold excess of cells ($10^6$) is required to obtain a significant inhibition.

#### *2. Cold Target Inhibition of the HLA-A2 and HLA-B7 Restricted Anti H-Y Cytotoxic Cells*

Figure 8 shows an example of the reduction in lysis by the addition of selected cold target inhibitors to the hot target cells. No significant inhibition was obtained by adding $1 \times 10^5$ nonrelevant inhibitors, i.e., non-A2, B7 blasts) whereas 72% reduction was observed by the addition of cold male target cells carrying both the required HLA-A2 and the B7 antigen. Intermediate reduction was observed after the addition of either HLA-A2 male target cells or HLA-B7 male target cells.

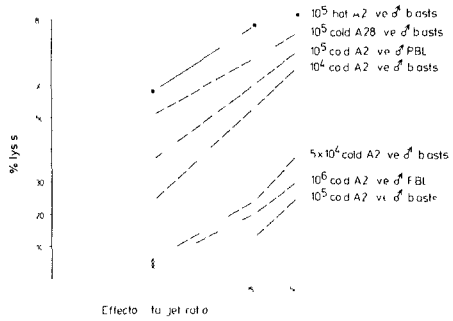


FIGURE 7 Specific inhibition by cold stimulated and unstimulated target cells to the hot target cells

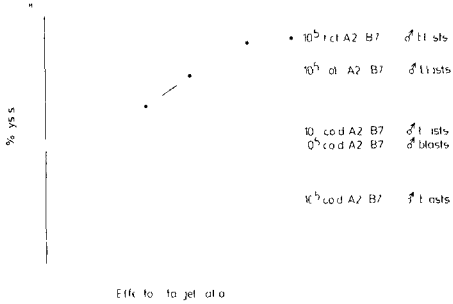


FIGURE 8 Reduction in lysis of the HLA A2 H Y/B7 H Y CTL by cold target cells

## D Long-Term Culture and Cloning Procedures

Many developments in culturing, expanding, and cloning of human T cells have been made since it was shown that such cells could be maintained in culture for prolonged periods of time (Svedmyr)[54] Long term culture of human T lymphocytes can be achieved by using conditioned medium (CM) or T cell growth factor (TCGF) both are derived from the supernatants of mitogen-stimulated lymphocytes as reported by several authors,[55-63] and now called Interleukin 2 (IL 2) The utilization of irradiated autologous lymphoblastoid cell line cells improves the growth conditions and increases the cell yield of the cultures (G Bonnard personal communication and Inouye et al [64]) Expansion and maintenance of human alloreactive T cell lines and cloning of either proliferative or cytotoxic populations also can easily be achieved by using lectins and irradiated feeder cells [65-69] Using the latter technique we have expanded, maintained, and cloned the HLA A2 restricted anti H Y cytotoxic T cells (Goulmy et al [70])

### *1 Long-Term Growth of the HLA-A2 Restricted Anti H Y Cytotoxic T Cell Lines*

Continuous growth of 6-day specific cytotoxic effector cells can be carried out by the regular additions of irradiated feeder cells (i e , the specific original stimulator cell)

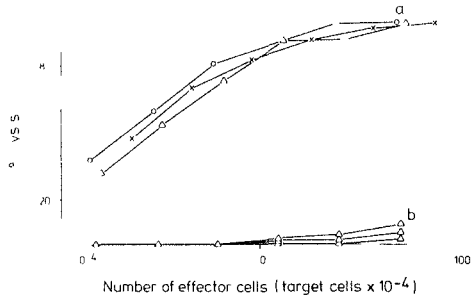


FIGURE 9 Specific anti HLA A2 H Y lysis by three cytotoxic T cell lines ○——○ △——△ ×——× represent different cytotoxic T cell lines with their lytic capacity against HLA A2 positive male target cells The lower lines in the figure (△ △) represent the amount of lysis respectively obtained against HLA A2 negative male target cells and HLA A2 positive and negative female target cells

and a lectin PHA 1 μg/mℓ of the two times crystallized leukoagglutinin Pharmacia Fine Chemicals cat no 17 0630 01 has been used in our studies The cytotoxic T cell lines are expanded in tissue culture flasks (Falcon no 3013) containing $2 \times 10^5$ effector cells per mℓ and are fed with $3 \times 10^5$ 5 000 rad irradiation feeder cells plus PHA Every third or fourth day growth has to be checked and the effector cells are diluted back to a starting number of $2 \times 10^5$ effector cells per mℓ and are fed Another useful technique for maintaining the cytotoxic T cell lines is the use of the commercially available TCGF (Lymphokult T Biotest Cat No 812800 an appropriate final concentration is 20% in the culture medium) With special regard to the cytotoxic T cell lines (showing HLA A2 restricted anti H Y cytolytic activity) we have found that optimal growth could best be obtained by utilizing both of the available procedures In our experience the maintenance of functional cytotoxic T cell lines requires not only TCGF but also repeated antigen presentation The cytotoxic T cell lines exert strong cytolytic activity after 3 weeks in culture (approximately 50% lysis in a 2 1 effector/target ratio) and they retain their anti male HLA A2 restricted specificity (see Figure 9) These cell lines can be maintained in culture for more than 6 months by the use of mitogen and irradiated feeder cells

*2 Cloning*

Several cloning procedures can be used A soft agar technique for separating subpopulations from MLC colonies (originally described by Rosenszjn et al [71]) is used by Zeevi et al [72] Cell cloning can be achieved after 1 × g sedimentation (as originally described by Miller and Philips[73]) thereafter plated at a dilution of 1 cell/well [64] Cloning can also be achieved by direct limiting dilutions from the long term cultures Limiting dilutions can be made in such a way that statistically one would expect e g , 10 cells, 1 cell or less than 1 cell per conical microtiter well This latter method has been used in our laboratory with success

Primary limiting dilutions on a 1 and 10 cell basis per conical microtiter well (250 μℓ volume) have been set up, and recloning has been performed as well The limiting dilutions were prepared from the cytotoxic T cell lines (CT lines) Growth promotion is provided by adding specific 3500 rad γ irradiated feeder PBLs ($1 \times 10^4$ original stimulator cells) and the lectin PHA (1 100 final dilution or Leucoagglutinin 1 mg/mℓ Pharmacia Sweden) A flow chart for our procedure is shown in Figure 10 After 10

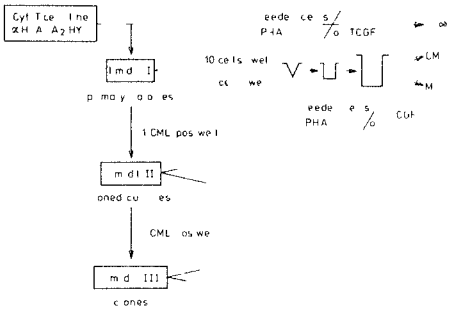

FIGURE 10 Flow chart for long term cultures cloning and recloning.

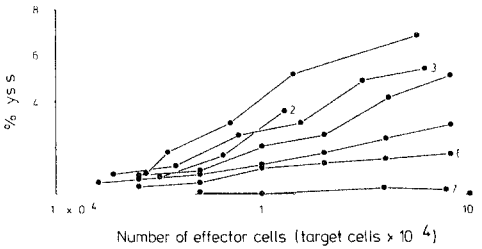


FIGURE 11 Lytic capacities of some primary colonies derived from the first limiting dilution against HLA A2 positive male target cells.

days, the contents of the wells are transferred to separate flat bottomed microtiter wells (250 $\mu\ell$ volume) followed by the addition of irradiated feeder cells ($3 \times 10^4$) and PHA. After 3 days, each well is screened through the use of a phase contrast microscope for the presence of growing colonies. When growth is observed the contents of the microwell are transferred to a 2 m$\ell$ cluster well. Culturing is continued using feeder cells ($5 \times 10^5$) and PHA as described above. As soon as enough cells can be harvested, they are tested for cytotoxicity. Figure 11 gives some examples of the lytic capacities of some primary colonies. The results indicate the variability in the levels of *specific* cytotoxicity. Several hypotheses can be proposed to explain this fan shaped phenomenon. One possibility is the existence of heterogeneous T cell receptors in the different primary colonies, each with varying affinities for the target cells or unequal lytic potentials of the primary colonies. Another explanation for the different lytic potentials could be the result of differences within the primary colonies in terms of growth synchrony. Possible objections against the proposed hypothesis is that one cannot refer to "clones" after only one limiting dilution. Therefore, we performed recloning of one primary colony (Figure 12, recloning has been done from the primary colony indicated by an asterisk). The results indicated an almost perfect fit with the earlier obtained lytic capacity, which indicates that, now that we have reached the stage in which one might more properly speak of cloning, there is little variation in lytic capacity from one clone to the other.

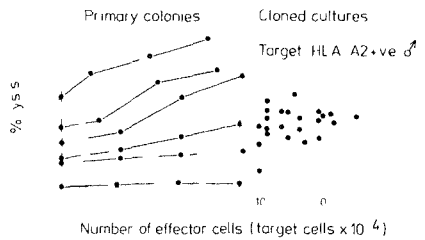


FIGURE 12 Lytic capacities of some recloned cultures originally derived by limiting dilution from the primary colony indicated by an asterisk and redilulted on a one cell basis

*3 Implications of Long Term Cultures and Cloning Techniques*

The possibility of maintaining human cytotoxic T cells in culture opens up new areas for investigation Homogeneous cloned cultures provide the opportunity for studying different subsets of cytotoxic T cells, and their membrane specific markers and/or receptors biological funtions, and biochemical characteristics

## III RELEVANCE IN CLINICAL MEDICINE

T cell recognition systems have been described in rodents and have been used to clarify T effector cell functions (i e , cell mediated cytotoxic responses towards virus and hapten modified cell surface antigens), Zinkernagel et al [10], Shearer et al [11] It is obvious that with the increasing amount of evidence on the functions of the highly polymorphic HLA supergene we have now come closer to understanding its active role in the immune process, and in all probability, its guidance of the HLA restricted cytotoxic T cell subpopulations, e g , in immunosurveillance against tumors and primary infections However, very little evidence has been presented on the presence of HLA restricted cytotoxic T cells during primary viral infections Nevertheless, the involvement of H 2 in primary viral infections has been established by Zinkernagel and Doherty[74] and Blanden[75]

The minor histocompatibility antigen H Y may be involved in graft rejection and acts as a strong transplantation antigen, as was most probably the case in our female bone marrow transplanted aplastic anemia patient This patient rejected an HLA identical male graft The presence of strong anti male cytotoxic effector cells and of an anti male HLA restricted IgM antibody (van Leeuwen et al [76]) are clearly in vitro reflections of the already established influence of sex in both rejection and graft vs host disease in bone marrow transplantation (Storb et al [77], van Rood et al [78])

The importance of the HLA A2 restricted H Y immunity in kidney transplantation was investigated in 1978 (Goulmy et al ) [79] A retrospective study (Eurotransplant patients) of cadaveric renal allograft survival at two years showed a significant difference between HLA A2 females receiving HLA A2 male kidneys and non HLA A2 females receiving non HLA A2 male kidneys The H Y incompatibility in donor recipient combinations sharing the HLA A2 antigen resulted in a 38% graft survival at two years in contrast to 58% in the HLA A2 non sharing group

We have recently described another example in which CML, as an in vitro model, can reflect in vivo processes (Goulmy et al ) [80] We have observed a significant correlation between the development of donor specific CML nonreactivity and good kidney

graft function Furthermore compatibility for the HLA-B locus antigens in the donor recipient pairs and sex-match (i e , a male recipient who received a male graft) predispose to the development of donor specific CML nonreactivity

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